GENETIC AND ENVIRONMENTAL EFFECTS ON DEVELOPMENTAL TIMING, OTOLITH FORMATION, AND GILL RAKER DEVELOPMENT IN PINK SALMON FROM AUKE CREEK, ALASKA

By

Dion Oxman

RECOMMENDED:

Dr. Milo Adkison

Dr. Gregor Cailliet

Dr. Peter Hagen

Dr. William Smoker

Dr. Anthony Gharrett
Advisory Committee Chair

Dr. Milo Adkison
Chair, Graduate Program in Fisheries Division

APPROVED:

Dr. Michael Castellini
Dean, School of Fisheries and Ocean Sciences

Dr. Lawrence Duffy
Dean of the Graduate School

April 5, 2012
Date

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A

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Dion Oxman, B.S., M.S.

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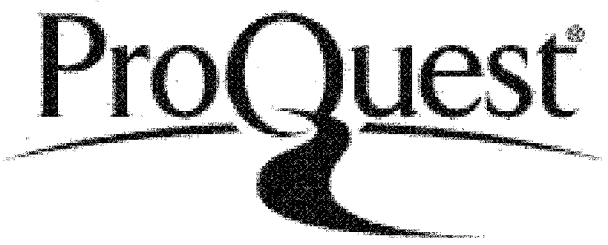

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ABSTRACT

To determine how inheritance, environment, and hybridization influenced developmental timing, otolith formation, and gill raker development in pink salmon (*Oncorhynchus gorbuscha*), full and half-sibling families from Auke Creek, Alaska and third generation outbred hybrids between Auke Creek females and Pillar Creek males from Kodiak Island, Alaska (1,000 km distant) were incubated in ambient, chilled, and warmed water. Variation in development time of embryos from the odd-year broodline was primarily influenced by additive genetic factors, whereas no genetic effect was detected in the even-year run. No genotype-by environment (GxE) effects were associated with sires or families in either broodline, indicating that the observed variation in development time was likely the result of phenotypic plasticity. Hybridization (outbreeding) significantly prolonged development time in both broodlines, indicating that the phenotypic effects of outbreeding can last at least three generations. Early otolith development was genetically conserved and canalized, but the phenotypic expression of these genes is plastic and strongly influenced by environmental factors. There was no evidence that local adaptation or outbreeding influenced otolith morphology or shape. Otoliths from fish exposed to thermal stress were bilaterally asymmetrical, whereas the bilateral symmetry of otoliths from outbred fish exhibited evidence of heterosis because they were more symmetrical than their native counterparts. Unlike development time and otoliths, gill raker development was linear and consistently stable in the face of both

hybridization and environmental stress. These results make it clear that different biological attributes respond to genetic control and stress in different ways.

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GENERAL INTRODUCTION

In philopatric species that have numerous local populations across a diverse range of environments, there is a potential for the genomes to become adapted to local conditions. The well documented homing ability of Pacific salmon (*Oncorhynchus* spp.) to their natal streams for spawning promotes reproductive isolation and restricts gene flow, thereby increasing the potential for such adaptation as genomes become adapted to local conditions (Taylor 1991). The large diversity in genetic structure, morphology, behavior, and life-history patterns that exists among populations of salmon have long been recognized as evidence of their adaptation to local environmental conditions and selection regimes (Ricker 1972, Taylor 1991,Verspoor et al. 2005). This evidence, however, is circumstantial.

To determine if a trait is locally adapted, it must be demonstrated that variation in the character under consideration has a genetic basis and that it enhances survival and/or reproduction for a deme while it is in its particular environment (Kawecki and Ebert 2004). One would therefore expect to see local differences in fitness-related traits such as growth rate, size, fecundity, homing ability, and temperature tolerance (Taylor 2004). Such evidence, however, is difficult to obtain. Instead, genotype-by-environment (GxE) interactions have been used to infer local adaptation (Lynch and Walsh 1998). Genotype-by-environment interactions occur when different genotypes respond to the same environmental change in different ways and have been associated with a variety of fitness-related traits in salmon including survival (Evans et al. 2010), development rate

(Hebert et al. 1998), and fin size (Taylor and McPhail 1985). However, because the spatially divergent selection responsible for GxE interactions and the creation of locally adapted traits can be hindered by gene flow, confounded by genetic drift, opposed by natural selection due to temporal environmental variability, and constrained by the genetic architecture underlying traits, local adaptation is not the only possible result of such selection (Kawecki and Ebert 2004). Consequently, the presence of G×E interaction by itself does not prove that a trait is locally adapted, but it does indicate that it may be predisposed towards such specialization (Kawecki and Ebert 2004).

The widespread distribution of salmon throughout the Pacific Rim and their tendency to return to their native streams to spawn not only encourages local adaptation, it makes their occurrence predictable in space and time. As a result, salmon fisheries often play an essential role in local and international economies. Management of these resources is difficult since local selection regimes have produced numerous, genetically distinct groups of fish (Hendry et al. 2000, Unwin et al. 2003, Crossin et al. 2004, Verspoor et al. 2005). This predilection towards local adaptation is a concern to fishery managers, especially in situations where native populations can interbreed with introduced fish stocks.

Hatcheries in Canada, Alaska, Russia, Japan, and Korea produce 5.2 billion salmon annually and release them into the Pacific Ocean to enhance fisheries, protect wild stocks, and supplement depressed populations (NPAFC 2011). There is an ongoing debate regarding the impact that these activities can have on wild salmon populations because of the genetic differences that can exist between wild and hatchery stocks (Naish

et al. 2007). Releasing cultured stocks into the environment essentially eliminates natural barriers to gene flow, thereby allowing non-native genes to become incorporated into the native genome. This introgression of foreign genes can alter locally adapted allele frequency distributions and gene complexes through a process called outbreeding depression (OBD), which may, in turn, reduce fitness and population viability (Chilcote et al. 2011, Frankham et al. 2011).

Both the additive and non-additive genetic effects of hybridization can result in outbreeding depression. If a trait is influenced by numerous independent genes, then mating genetically dissimilar parents can disrupt this additive influence and produce hybrid offspring that have phenotypes that are typically midway between the parental values (Falconer and Mackay 1996). These phenotypes would be expected to appear in the first hybrid generation, impart a reduced fitness in either parental environment, and persist until natural selection reduced the number of deleterious alleles (Lynch and Walsh 1998). In instances when a trait is influenced by an epistatic network of loci, outbreeding can alter phenotypes and reduce fitness by breaking up these coadapted gene complexes (Emlen 1991). Such changes may not appear in the first hybrid generation because each offspring receives a complete copy of the coadapted genome from each parent, but they will likely occur in the second hybrid generation and later, when parental genomes undergo independent assortment and recombination (Lynch and Walsh 1998).

Although both types of OBD can persist for several generations, few studies have examined its effects in salmon beyond the first or second generation (Gilk et al. 2004, Granath et al. 2004, Wessel et al. 2006, Dann et al. 2010); the long term impact on fitness

remains unknown. Pink salmon (*Oncorhynchus gorbuscha*) provide a good model for evaluating the long-term effects of outbreeding in salmon. They have a short, 2-year, semelparous, anadromous life cycle that has produced genetically distinct odd- and even-year broodlines, even in areas where these broodlines occur sympatrically (Beacham et al. 1985). Outbreeding in pink salmon has adversely influenced fitness-related traits including marine survival (Gilk et al. 2004), size (Gharrett and Smoker 1991), and developmental timing (Wang et al. 2007) in the first or second generation. Developmental timing is especially important to salmon fitness because each life stage must occur when environmental conditions are optimal for growth and survival (reviewed by Heard 1991). Because these conditions vary spatially, and since salmon return to their natal stream to spawn, the resulting differences among populations in selection pressure mean that developmental timing is often considered a locally adapted trait (Goddard 1995, Hebert et al. 1998). Environmental conditions can also vary temporally within locations, so such adaptation must also be sufficiently flexible to accommodate a specific range of environmental variation. For these reasons, development time is an ideal trait for studies of local adaptation and OBD in salmon.

In Chapter 1, I explore the possibility that developmental timing in pink salmon could be locally adapted and evaluate the long-term impact of outbreeding. Several steps were taken to accomplish these goals: (1) full- and half-sib families of Auke Creek (Juneau, Alaska) pink salmon embryos were incubated in ambient Auke Creek water to determine if the rate of development had a genetic basis; (2) subsets of these families were also reared in chilled and warmed water to determine if developmental timing was

influenced by GxE interactions which, if present, could be used to infer the presence of local adaptation; and (3) third generation hybrids between spatially separated populations were bred to evaluate the long-term effects of outbreeding.

In Chapter 2, I explore the effects of environment, inheritance, and outbreeding on otolith development. Otoliths are dense structures composed of calcium carbonate that are found in pairs in the inner ear of teleosts whose function is associated with hearing, balance, and orientation. Each individual possesses three pairs of otoliths, the largest of which are the sagittae, which grow incrementally and continuously throughout the life of an individual. Like developmental rate, otoliths can be considered a fitness-related trait because they help a fish perceive and interact with its environment and are, therefore, essential for survival. The size, shape, microstructure, and chemical composition of sagittal otoliths can vary significantly within a species; and this variation has been used to identify stocks (Bergenius et al. 2006), reconstruct life histories (Campana and Casselman 1993), and separate stocks in mixed stock fisheries (Begg et al. 2001).

Intraspecific variation in otolith morphology exists because its development is sensitive to biological and environmental change. The physiological changes associated with hormone cycles and aging, for example, can substantially alter an otolith's formation rate (Beckman and Wilson 1995, Campana 1999). The ectothermic nature of fish metabolism makes otolith formation particularly susceptible to environmental change, especially water chemistry and temperature (Savoy and Grecco 1987, Bestgen and Bundy 1998). Since growth is a metabolic process and since such processes are regulated by genes, some degree of genetic control over otolith development is also expected. This

expectation is even higher among salmonids, given their propensity for local adaptation. Little, however, is known about the mechanisms of this control, nor is it known if any of the observed phenotypic variation in otolith morphology derives from genetic sources.

Using the approach described in the first chapter, my goal in Chapter two was to determine how parental genomes influence otolith development. We also explored how environmental change and hybridization (outbreeding) influenced otolith formation to provide evidence for GxE effects (local adaptation), canalization, and/or outbreeding depression.

The physiological stress that results from exposure to environmental and genetic change (hybridization) can disrupt developmental pathways and produce phenotypic variability (Waddington 1942). Because stress promotes excess energy dissipation, compensatory energy expenditures may be made at the expense of the canalization process (homeostasis) to complete development. The resulting instability can disrupt genetic expression beyond the typical range of plasticity and generate high levels of phenotypic variation (Valentine et al. 1973). In bilaterally symmetrical organisms, this developmental instability may be expressed as asymmetry and measured in terms of "fluctuating asymmetry" (FA), a variable that describes structural deviation from perfect symmetry (Palmer 1994). Fluctuating asymmetry is often used to detect the effect of stress on developmental stability and population health (Palmer and Strobeck 2003).

Although environmental stressors such as pollution and climate change cause FA in fishes (Valentine et al. 1973, Alados et al. 1993), the impact of genetically-derived stress on developmental stability is less clear. For example, some studies indicated that

increased homozygosity caused by inbreeding was correlated with FA (Leary et al. 1985, Crozier 1997), whereas others had indicated that no such correlation exists (Panfili et al. 2005, Fessehaye et al. 2007). Similar ambiguity has been observed regarding the effects of outbreeding on bilateral symmetry (Wilkins et al. 1995, Gharrett et al. 1999). The nature of the relationship between genetic stress and development remains unclear and is obviously in need of further investigation, especially among salmon populations for which enhancement programs increase the risk of inbreeding and outbreeding.

Most bilateral structures can potentially be used to measure FA and evaluate developmental stability. Otoliths would seem to be particularly well suited for this task since their formation is influenced by both biological and physical factors (Campana 1999). Otolith FA has been correlated with stress in a variety of species including Pacific hake (*Merluccius productus*; Alados et al. 1993), Indian mackerel (*Rastrelliger kanagurta*; Al-Mamry et al. 2011), and European anchovy (*Engraulis encrasicolus;* Kristoffersen and Magoulas 2010). Stress in other species, however, such as the Atlantic menhaden (*Brevoortia tyrannus*; Fey and Hare 2008), Bonga shad (*Ethmalosa fimbriata*; Panfili et al. 2005), and Blackchin tilapia (*Sarotherodon melanotheron*; Panfili et al. 2005) had no detectable effect on otolith FA. Clearly the relationship between stress and otolith FA does not exist in all species and must be evaluated on a case by case basis before it is used as an indicator of developmental stability.

In Chapter three, I used the left and right otoliths from native and hybrid pink salmon reared in natural and controlled temperatures as described in the previous chapters to determine if environmental or genetic stress influenced otolith symmetry and,

if so, to evaluate the usefulness of otolith FA as an indicator of developmental stress. Comparisons between natives and outbred hybrids also allowed us to clarify the relationship between genetic stress and developmental stability.

The progressive growth of developmentally stable morphological traits that exhibit little variation under diverse environmental and genetic conditions are often used to identify, describe, and categorize developmental states, especially in species like Pacific salmon, which have complex life histories. In salmon, embryonic stages have been described by using Ballard's scale (Ballard 1973), which consists of 30 stages divided among three developmental phases: cleavage (cell division), epiboly (tissue formation), and organogenesis (organ formation), all of which precede the emergence of the yolk-bearing larva from the egg. Gross morphology and behavior are used to define those developmental milestones that occur after yolk absorption: free-swimming embryos, exogenously feeding alevin, seaward migrating smolt, marine phase juvenile, and anadromous adult stages (Groot and Margolis 1991). With the exception of yolk weight, however, no physical markers have been described that categorize the developmental stages that occur between hatching and emergence (Malecha 2002). Given that gill raker counts are used to characterize and identify species (Humphries 1993, Yokogawa and Seki 1995), we believed that the progression of raker formation in embryos could serve as a post-hatch developmental marker, provided that its development is unaffected by environment or genetic variation.

In the fourth and final chapter, I tracked the developmental progression of gill rakers in native post-hatch pink salmon embryos incubated in their native waters as well

as in genetically and environmentally stressed post-hatch embryos. This approach allowed us to determine if gill rakers grew in a predictable sequence and assess their developmental stability – both characteristics that are needed in order for a trait to be useful as a developmental marker.

A list of commonly used acronyms and terms is included at the end of this introduction for informational purposes (Appendix 1).

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APPENDIX 1
Glossary of Commonly Used Terms

Accumulated Thermal Units (ATU): a unit of measurement that quantifies thermal exposure over time; one ATU is equal to one degree Celsius for one day.

Additive Genetic Effects: when the combined effects of alleles at different loci are equal to the sum of their individual effects.

Allele: an alternative form of the same gene at a particular gene locus.

Aragonite: a crystalline form of calcium carbonate ($CaCO_3$) commonly found in sagittal otoliths of bony fishes.

Canalization: the processes associated with the production of consistent phenotypes regardless of variability associated with genotype or environment.

Fitness: an individual's contribution, relative to other individuals, to the breeding population in the next generation.

Fluctuating Asymmetry (FA): the deviation from perfect bilateral symmetry caused by biological and environmental stress.

Genotype-by-Environment (GxE) Interaction: a phenomenon in which different genotypes (families) respond to environmental change in nonparallel and unpredictable ways.

Heterosis: the improved or increased function of any biological quality in a hybrid offspring. Also referred to as hybrid vigor.

Hybrid Vigor: the improved or increased function of any biological quality in a hybrid offspring. Also referred to as heterosis.

Local Adaptation: genetic specialization within a population that has occurred in response to geographically localized selection pressures.

Locus: the location of the gene on a chromosome.

Midhatch: the number of days or accumulated thermal units 50% of embryos within an incubation compartment have hatched.

Outbreeding Depression (OBD): a reduction in population fitness that can occur when members of genetically dissimilar populations interbreed. This reduction can occur when

locally adapted allele composition and gene complexes become disrupted by the introgression of non-native genes.

Phenotype: the physical characteristics of an organism.

Phenotypic Plasticity: the ability of a phenotype to vary as a result of environmental influences on its genetic makeup.

Restricted Maximum Likelihood (REML): a statistical method for partitioning variation.

Vaterite: a crystalline polymorph of calcium carbonate ($CaCO_3$) that is found in the abnormal sagittal otoliths of bony fishes.

CHAPTER 1

GENETIC AND ENVIRONMENTAL EFFECTS ON DEVELOPMENT TIME IN EVEN AND ODD BROODLINES OF PINK SALMON (*ONCORHYNCHUS GORBUSCHA*) AND THEIR THIRD GENERATION OF OUTBRED HYBRIDS[1]

[1]Oxman, D.S., Smoker, W.W., and Gharrett, A.J. In Preparation. Genetic and environmental effects on development time in even and odd broodlines of pink salmon (*Oncorhynchus gorbuscha*) and their third generation of outbred hybrids. Canadian Journal of Fisheries and Aquatic Sciences.

ABSTRACT

Full and half-sibling families of odd and even broodlines of pink salmon from Auke Creek, Alaska and of third generation outbred hybrids between Auke Creek females and Pillar Creek males from Kodiak Island, Alaska (1000 km distant) were incubated in ambient, chilled, and warmed Auke Creek water to determine how inheritance, environment, and outbreeding influenced development timing. Additive genetic factors played a role in the variation of development time of embryos from the odd-year broodline, but they did not affect development time in the even-year run. No genotype-by environment (GxE) effects were associated with sires or families in either broodline, indicating that the observed variation in development time was likely the result of phenotypic plasticity. Outbreeding significantly prolonged development time in both broodlines and it altered the proportions of additive and environmental variation possibly by influencing the canalization process. The apparent outbreeding depression in these hybrids of geographically separated populations demonstrated that introgression of nonnative fish may erode fitness by altering locally adapted traits; and that these effects can last at least three generations, a potential concern to some aquaculture and enhancement programs.

Keywords: salmon, development, inheritance, local adaptation, outbreeding, plasticity

INTRODUCTION

Pacific salmon occupy a wide range of habitats at spawning, and their life histories are characterized by their tendency to return (e.g. "home") faithfully to natal habitats at maturity. Evolutionary genetics theory predicts that such exposure to local differences in selection pressure, in combination with the restricted gene flow associated with homing, will result in adaptation to local conditions and genetically distinct demes (Taylor 1991, Kawecki and Ebert 2004). Indeed, genetic divergence among stocks within populations of Pacific salmon has been documented by comparisons of biochemical genetic traits (e.g. Kondzela et al. 1994, Verspoor et al. 2005) and observations of polygenic phenotypic differences between ecologically adaptive traits (e.g. Unwin et al. 2003, Crossin et al. 2004). This local adaptation and the potential for the practice of artificial culture of salmon to disrupt it have raised concern (Naish et al. 2007).

Hatcheries throughout the Pacific Rim produce and release salmon to enhance fisheries, protect wild stocks, and supplement depressed populations (Naish et al. 2007). These activities, however, can remove natural barriers to gene flow. Increased gene flow can make conservation of natural diversity difficult because cultured stocks can differ genetically from their wild spawning counterparts (Naish et al. 2007); and if members of genetically dissimilar populations interbreed, a reduction in population fitness can occur as locally adapted allele composition and gene complexes become disrupted by the introgression of non-native genes through outbreeding depression (OBD; Lynch and Walsh 1998).

Outbreeding depression can result from both the additive and non-additive genetic effects of hybridization. For traits influenced by additive genetic variation, outbred individuals may have phenotypes that are midway between the parental values (Lynch and Walsh 1998). These phenotypes would be expected in the first outbred generation and may impart reduced fitness in either parental environment (Lynch and Walsh 1998). When OBD influences phenotypes by breaking up coadapted gene complexes, a non-additive effect, changes may not appear until the second hybrid generation and later after parental genomes have undergone independent assortment and recombination (Lynch and Walsh 1998). Although both types of OBD would be expected to persist for several generations (Gharrett et al. 1999), most studies of salmon have only examined the effects in the first or second generation (Gilk et al. 2004, Granath et al. 2004, Wang et al. 2006, Dann et al. 2010).

Pink salmon (*Oncorhynchus gorbuscha*) provide a good model for evaluating the long-term impact of outbreeding in salmon. They have a short, two-year, semelparous, anadromous life cycle that has produced genetically distinct odd- and even-year broodlines, even in areas where these broodlines occur sympatrically (reviewed by Groot and Margolis 1991). Outbreeding in pink salmon has been observed to adversely influence fitness-related traits including marine survival (Gilk et al. 2004), size (Gharrett and Smoker 1991), and developmental timing (Wang et al. 2007) in the first or second generation. Developmental timing is especially important to salmon fitness because larval stages must occur when conditions of their seasonally labile high-latitude habitats are optimal for growth and survival, particularly in terms of water temperature and food

availability (reviewed by Heard 1991). Because these habitats vary spatially and salmon return to their natal stream to spawn, developmental timing is often a locally adapted trait (Goddard 1995, Hebert et al. 1998). Environmental conditions can also vary temporally within locations, so such adaptation must also be sufficiently flexible to accommodate to a range of environmental variation. For these reasons, development time is an ideal trait for studies of local adaptation and OBD in salmon.

The primary objective of our study was to confirm that developmental timing in odd and even broodlines of pink salmon could be locally adapted, as well as to evaluate the multi-generational effect of experimentally induced outbreeding. Several steps were taken to accomplish these goals: (1) full- and half-sib families of Auke Creek (Juneau, Alaska) pink salmon embryos were incubated in ambient Auke Creek water to determine if developmental timing had a genetic basis; (2) some of these families were reared in chilled and warmed water for the first 14 weeks to determine how differences in the thermal environment early in development influenced genotypic expression because local adaptation can be inferred from genotype-by-environment (GxE) interactive effects (Lynch and Walsh 1998); and (3) third generation hybrids between spatially separated populations ("spatial hybrids") were cultured to evaluate the long-term effects of outbreeding. We replicated the experiments in the genetically isolated odd- and even-year populations of pink salmon at Auke Creek.

MATERIALS AND METHODS

Breeding and Incubation

Pink salmon were collected at Auke Creek Research Station (a facility of US National Oceanic and Atmospheric Administration's Alaska Fisheries Science Center) from a weir near the mouth of Auke Creek, a 350 m high-gradient, lake-fed stream located near Juneau, Alaska. Auke Creek has naturally spawning populations of pink salmon. Mature salmon used in this experiment were fin-marked individuals which represented the second filial (F_2) generation of a breeding experiment that involved native Auke Creek pink salmon and spatial hybrids that had been released as fry into the North Pacific Ocean from the Auke Creek Research Station during the spring of 1999 and 2000. The hybrid lineage was originally created by crossing late-run females in Auke Creek with late-run males from Pillar Creek on Kodiak Island, Alaska (Gilk et al. 2004). Pillar Creek, a 1800 m long reservoir-fed stream located 1000 km west of Juneau, is historically 1 to 2 °C cooler than Auke Creek between late August when the salmon spawn and mid-November when their eggs hatch. Although the latitudes (near 58°N) and habitats of Auke and Pillar Creeks are similar, their spatial separation and different temperature regimes make it likely that the groups have evolved independently into genetically distinct locally adapted populations (Adkison 1995, Gilk et al. 2004). Consequently,

crosses between these demes produced spatial hybrids that allowed us to study the effect of outbreeding on development time.

In late summer 2000 and again in 2001, we conducted two replicate breeding experiments. The F_2 generation of maturing salmon, descendants of even-year natives and hybrids bred in 1996 by Gilk et al. (2004) and odd-year natives and hybrids bred in 1997 by Wang et al. (2007), were collected as they returned to Auke Creek, sorted according to gender and ancestry (native or hybrid, identified by fin clip), and held for several days until maturation was complete. On 3 September 2000, a blocked 2x2 factorial design was used to produce full- and half-sib F_3 families from native (20 males and 20 females) and hybrid (16 males and 16 females) returning fish from the even-year broodline (BY 2000). There were 10 blocks of natives (40 families) and 8 blocks of hybrids (32 families). On 8 September, 2001, the same design was used to produce offspring from native (32 males and 32 females) and hybrid (20 males and 20 females) fish from the odd-year broodline (BY 2001). This produced 16 blocks of natives (64 families) and 10 blocks of hybrids (40 families). All crosses were conducted within years.

During both experiments, fertilized eggs were incubated in divided trays (FAL^{TM}; Marisource, Milton, WA) that housed each family separately. Each tray had 10 compartments which contained single families. These trays were placed in incubation cabinets so that each cabinet contained eggs from a single type of cross (native or hybrid). The eggs in one native and one hybrid cabinet were incubated in ambient temperature Auke Creek water. Because water temperatures in the stream, intragravel environment (redds), and research station are similar, development of natives reared

under ambient conditions represented the normal development of wild Auke Creek pink salmon. These natives were the control group. A subset of eggs from native BY 2000 families were placed in a 3rd cabinet and incubated in a simulated environment that was, on average, 2.4 °C (± 0.9 °C) cooler per day than ambient Auke Creek stream temperatures through hatching (Fig. 1.1). During the BY 2001 experiment, incubation temperatures in the simulated environment were 2.4 °C (± 0.8 °C) warmer than ambient stream temperatures through hatching (Fig. 1.1). All families were divided into four approximately equal portions, two of which were randomly assigned to compartments within a cabinet in each temperature regime to provide replication. This experimental design produced three treatment groups within each brood year: ambient natives (controls: AC), cold natives (CC), and ambient hybrids (AH) during brood year 2000, and AC, warm natives (WC), and hybrids (AH) during 2001.

The temperature in the simulated environment was slightly altered daily to mimic natural variation in incubation conditions (Fig. 1.1). Water in both simulated environments was re-circulated to achieve the level of control needed to simulate daily thermal fluctuations in a captive setting. Two 1,136-liter Living Streams (Frigid Units, Inc. Toledo, OH) were used as a reservoir and equipped with a 1-hp chiller (BY 2000) and two aquarium heaters (BY 2001). A ¾-hp submersible pump was used to move water from the reservoir to a 76-liter head tank, which distributed water to the incubation cabinets by gravity feed. Water from the incubators drained into the reservoir, where it was conditioned and pumped back to the head tank. Water in the reservoirs was

continually replaced with fresh stream water; a complete turnover occurred every 5 hours.

Water was supplied to all incubators at a rate of 8 L/min until the eyed stage was reached and 23 L/min thereafter. Temperatures were recorded daily to the nearest 0.1 ^{o}C and did not differ substantially between incubation cabinets for a particular temperature regime. Before hatching, incubating eggs were treated once a week with formalin (1:6000 in static water) for one hour to reduce infestations by fungus and algae, a standard practice in the culture of salmon embryos (Wood 1968). After hatching, weekly salt treatments (3 parts per thousand sodium chloride for 1 hour) were used to minimize infestations, another common practice in salmon culture. All water was filtered through sheer nylon mesh prior to entry into the incubation cabinets to prevent hydra infestation.

Observations of Embryonic Development

Larval development time, the midhatch date, defined as the first day on which 50% or more of the eggs in a compartment had hatched, was determined for each replicate group of embryos. Starting before hatching began (early November) and continuing until hatching was complete (mid-December), daily observations were made of the number of eggs that had hatched in each compartment. Development time was measured in terms of days and accumulated thermal units (ATU; a unit of measurement that quantifies thermal exposure over time; one ATU is equal to one degree Celsius for one day).

Statistical Analysis

Restricted maximum likelihood analysis (REML) was used to determine how parents, incubation temperature, and spatial hybridization influenced variation in developmental timing. Restricted maximum likelihood is more robust to deviations from normality, non-homogenous variances, and unbalanced designs in mixed-model comparisons than ANOVA (Lynch and Walsh 1998, Van Dongen et al. 1999). The REML method uses the *Z*-statistic, defined as the estimated covariance parameter divided by its approximate standard error, to test random effects for significance. The *F*-statistic is used to test fixed effects. Development time was compared among families within a treatment with the following REML model:

$$(1) \quad Y_{klmno} = \mu + B_k + D_{kl} + S_{km} + D_{kl}*S_{km} + R_{klmn} + \varepsilon_{klmno}$$

where Y_{klmno} was the dependent variable (number of ATUs or Days to midhatch.), μ the population mean, B_k the block effect (i.e. the independence and randomness of the experimental design), D_{kl} the dam effect, S_{km} the sire effect, $D_{kl}*S_{km}$ the interaction between dam and sire (i.e. family effects), R_{klmn} the effect of compartment position on development (i.e. microhabitat effects), and ε_{klmno} the residual random error (i.e. variation within families). All factors in this model were random.

The following REML model was used to test the effects of temperature on development time:

$$(2) \quad Y_{jklmno} = \mu + T_j + B_k + T_j*B_k + D_{kl} + S_{km} + D_{kl}*S_{km} + T_j*D_{kl} + T_j*S_{km} +$$

$$T_j{*}D_{kl}{*}S_{km} + R_{jklmn} + \varepsilon_{jklmno}$$

where T_j was the effect of incubation temperature, $T_j{*}B_k$ the interaction between temperature and block (i.e. the effect of temperature on the randomness and independence of the experimental design), $T_j{*}D_{kl}$ the effect of the interaction between temperature and dam (i.e. environmental interactions related to dam effects), $T_j{*}S_{km}$ the effect of the interaction between temperature and sire (i.e. GxE interactions related to sire effects), and $T_j{*}D_{kl}{*}S_{km}$ was the interaction between temperature and the family effect (i.e. GxE interactions related to family effects). The equation used to determine if spatial hybridization influenced development time was defined as:

$$(3) \quad Y_{iklmno} = \mu + C_i + B_{ik} + D_{ikl} + S_{ikm} + D_{ikl}{*}S_{ikm} + R_{iklmn} + \varepsilon_{iklmno}$$

where C_i was the effect of cross. Equations 2 and 3 were mixed-models because they contained both random (block, dam, sire, replicates) and fixed (cross, temperature) effects.

Another potential result of developmental instability that may arise from either genetic or thermal stress is increased phenotypic variation (Palmer and Strobeck 2003). Therefore, the variation associated with each otolith measurement was also compared among treatments with Levene's test for homogeneous variances to determine if treatment increased phenotypic variability, which would be considered an indicator of disrupted development and outbreeding depression.

Because the relationships within and among families were known, we used a quantitative genetics approach to express the variability partitioned by each REML model in terms of its underlying causal genetic components (Lynch and Walsh 1998). Given our

breeding design, the variation in development time associated with sire effects would imply that the heritable source of such variability came mostly from additive genetic factors (Table 1.1). Dam-associated variability also would primarily provide evidence of an additive effect, as well as for maternally inherited effects such as common maternal environmental influences, which includes egg quality and egg size (Table 1.1). Interactions between dam and sire would imply that variation in development time came from non-additive genetic sources such as dominance and/or epistatic effects (Table 1.1). Therefore, by using REML to test for significant dam, sire, and family effects, we evaluated the underlying genetic components of variation on development time.

Our experimental design also allowed us to determine if development time was potentially influenced by locally adapted genes. By rearing members of each native family in two thermal environments, we were able to use REML (model 2) to determine if GxE interactions occurred between dam and temperature, sire and temperature, and family and temperature. Significant GxE interactions would mean that different genotypes (different families) responded to environmental change in different ways and such responses indicated that development time had the potential to be locally adapted (Lynch and Walsh 1998). We could only suggest that it was locally adapted because proof would have required evidence of enhanced survival and/or reproduction for the deme in its native environment (Kawecki and Ebert 2004). The absence of GxE interactions would indicate that genotypes responded similarly to environmental change and that any observed differences in development time were probably caused by phenotypic plasticity rather than genetic differences. Because GxE effects associated with

dams are confounded by common environmental effects such as egg quality and egg size, only GxE effects associated with sires and families were used to evaluate development time for local adaptation.

All comparisons and hypothesis tests were conducted in SAS 9.1with PROC MIXED procedures (SAS Institute, 2002). The significance level used for hypothesis testing was $p \leq 0.10$. Native embryos reared at ambient temperature (controls) were used to establish the normal range of development time and variability of the reference population (i.e. Auke Creek pink salmon). Differences in development time among native embryos incubated in cold and warm water relative to that of natives raised at ambient temperature was presumed to indicate that developmental processes were thermally influenced. Similarly, differences observed in development time of spatial hybrids relative to that of controls were presumed to reflect the genetic differences between hybrids and natives and indicative of outbreeding.

RESULTS

Native Auke Creek Embryos (Controls)

Midhatch times observed among native families provided baseline information about development time and inheritance mechanisms for even- and odd-year broodlines of native Auke Creek pink salmon. In the BY 2000 experiment, AC embryos reared in ambient temperature creek water reached midhatch after 63.3 (± 1.0) days which was

609.6 (± 6.9) ATUs (Fig. 1.2). Comparisons among families indicated that neither sire, dam, nor interaction between dam and sire affected midhatch timing in terms of days or ATUs (Table 1.2).

The development and inheritance patterns of odd-year embryos differed from those of their even-year counterparts. In the BY 2001 experiment, AC offspring achieved midhatch after 66.9 (± 1.2) days [612.1 (± 5.7) ATUs; Fig. 1.3]. Unlike the even-year line, timing was influenced by genetic sources. Both dam and sire effects influenced midhatch timing as defined by number of days and ATUs (Table 1.2), which implied that additive inherited genetic factors and possibly maternal effects played a significant role in developmental timing. Interactions between dam and sire did not affect variation in midhatch timing (Table 1.2), which indicated that non-additive genetic variation (e.g. dominance and epistatic effects) had little influence on development time.

A family's position within an incubation tray influenced development in both experiments ($p \leq 0.001$: Table 1.2). Midhatch occurred 0.44 (± 0.33) days later than average in native families that were located at the front of a tray farthest from the water source, whereas it occurred 0.55 (± 0.34) days earlier than average in families toward the middle of a tray. Other positions within a tray had little effect on development; midhatch dates in these compartments differed from their overall means by less than 0.06%. Similar patterns of positional influence were observed within incubation trays from the other treatments (Table 1.2). Because the changes produced by these positional effects were small, and because families were randomly assigned to compartments, this variation

would contribute to the background noise but not directly influence the effects being tested.

Temperature Effects

Native offspring incubated in ambient temperature Auke Creek water were compared to those reared under experimental conditions to determine if the thermal environment influenced development time and to evaluate GxE interactions. In the BY 2000 experiment, development of embryos reared in cold water was significantly slower than that of embryos raised at ambient stream temperatures ($p \leq 0.001$; Table 1.2); it took 28.5 (± 1.2) more days for CC embryos to reach midhatch, but they achieved that milestone with 68.9 (± 5.3) fewer ATUs (Fig. 1.2). Genotype-by-environment effects were not associated with dam, sire, or interaction between dam and sire (Table 1.2), indicating that the observed variation in development time was likely a result of phenotypic plasticity. Although dam and sire effects were not detected among embryos reared in ambient temperature water, these effects were significant among embryos incubated in colder waters, which suggested that the cooler temperatures increased the proportion of additive genetic variation associated with development time (Table 1.2).

Levene's test indicated that the variability associated with development time in the BY 2000 experiment was influenced by temperature; variation of the number of days to midhatch increased by 34.2% in the cold regime ($p \leq 0.001$), and the variation of ATUs to midhatch decreased by 68.7% ($p \leq 0.001$).

In the BY 2001 experiment, native eggs reared in warm water had increased development rates relative to natives reared at ambient creek temperatures ($p \leq 0.001$; Table 1.2); the WC embryos achieved midhatch 11.8 (± 1.0) days earlier than their ambient counterparts (AC), but they did so after exposure to 54.9 (± 8.9) more ATUs (Fig. 1.3). Significant GxE effects were associated with dams ($p = 0.098$), but no GxE effects were associated with sires or families (Table 1.2) which suggested that variation in development time was mostly caused by phenotypic plasticity.

The accelerated rate of development in the BY 2001 experiment had no effect on the variation in the number of days to midhatch ($p = 0.414$), but it did increase the variability of ATUs to midhatch by 195.1%. ($p \leq 0.001$).

Hybridization Effects

The development rate of native pink salmon incubated at ambient temperatures was compared to that of the third generation of their spatial hybrids reared under the same conditions to evaluate the long term effects of outbreeding on development time and inheritance mechanisms. Midhatch timing in the even-year run was significantly altered by outbreeding ($p \leq 0.001$; Table 1.2); BY 2000 AH embryos needed 6.7 (± 1.2) more days and 42.5 (± 7.5) more ATUs to reach midhatch than their native counterparts (Fig. 1.2). Levene's test indicated that the variation in development time, however, did not differ between crosses, either in terms of days ($p = 0.869$) or ATUs ($p = 0.295$). Outbreeding also changed how genes influenced developmental timing. Despite the fact

that neither dam nor sire effects were observed among native families, they both exerted a significant effect on midhatch timing in hybrid embryos (Table 1.2). The absence of interaction between dam and sire that was evident among AC families, however, was also observed in AH families (Table 1.2).

Outbreeding also altered midhatch timing and its genetic controls in the odd-year run ($p \leq 0.001$; Table 1.2); midhatch in BY 2001 hybrids occurred 2.2 (± 1.7) days later than in the AC embryos, and they needed 10.6 (± 8.4) more ATUs to do so (Fig. 1.3). Levene's test indicated that the variability of this timing, however, was similar between crosses both in terms of days ($p = 0.165$) and ATUs. ($p \geq 0.230$). Outbreeding influenced genetic mechanisms as well by altering the affects of the sire's genome. Sires, which exerted additive control over midhatch timing in native offspring, had no significant affect on timing in hybrid offspring (Table 1.2). Dam effects, in contrast, were unaffected by the spatial hybridization, and family effects had no significant influence in either cross (Table 1.2). This apparent outbreeding depression in spatial hybrids suggests the introgression of nonnative fish may erode fitness by altering locally adapted traits, and that these effects can last at least 3 generations.

DISCUSSION

Although odd and even-year broodlines of pink salmon in Auke Creek have evolved in the same or very similar average environments, they have apparently evolved into two genetically distinct populations, at least in terms of the allelic composition

associated with development rate. This genetic difference was observed phenotypically; it took embryos from the even-year broodline approximately four fewer days to reach midhatch than their odd year counterparts despite the fact that a similar amount of ATUs were needed to achieve midhatch in each broodline (the average number of ATUs to midhatch differed by only 2 ATUs between broodlines). It is unlikely that this difference was caused by differences in incubation temperature because the average daily temperature in Auke Creek was similar between years (it differed by only 0. 4° C)

Quantitative genetic analysis confirmed that phenotypic differences in developmental timing had a genetic basis and that this basis differed between the odd- and even-year populations. Paternal and maternal control over midhatch timing in the odd-year broodline existed primarily through additive genetic inheritance, and possibly maternally inherited factors. Similar observations were reported by Hebert et al. (1998) and Goddard (1995). In contrast, sire and dam effects exerted little influence over such timing in the even-year broodline, indicating that heritable controls were conserved in that run. The absence of additive control over development time in this run was also observed by Joyce (1986), and it was reported by Goddard (1995). The lack of additive genetic variance may occur because natural selection can act to remove genetic variability from traits whose phenotypes are closely related to fitness (Fisher 1958). Genetic differences between sympatric runs of pink salmon have been observed previously (Aspinwall 1974, Beacham et al. 1985) and may have been caused by the reproductive isolation that results from their two-year life cycle, random genetic drift within broodlines, differing origins of the founder stocks, differing lengths of

colonization, or different environmental sequences, but the simplest explanation for quantitative genetic data is that each broodline has found different genetic solutions for adapting to the same environment (Gharrett et al. 1999).

Changes in incubation temperature within a broodline strongly influenced the phenotypic expression of genes controlling development timing. Low incubation temperatures prolonged development during BY 2000, whereas high temperatures shortened development times during BY 2001. This thermal disruption of developmental pathways was not unexpected because fish are ectothermic and, as such, their metabolic processes are regulated to some degree by their external environment.

Development time has often been considered to be locally adapted in pink salmon (Goddard 1995, Hebert et al. 1998, Gharrett et al. 1999, Taylor 2004). In this experiment, however, no GxE effects were associated with incubation temperature and sire or incubation temperature and family in either broodline; development time of individual families (i.e. genotypes) within each brood year typically responded to thermal change in similar and predicable ways. This is similar to observations made by Joyce (1986). Although GxE effects were associated with dams in the odd-year run, this interaction could not be used to evaluate development time for local adaptation because the effect is confounded by maternal factors such as egg quality and egg size. Consequently, most of the observed variation in development rate was likely the result of phenotypic plasticity. From an evolutionary perspective, phenotypic plasticity is important because it can set the stage for local adaptation by helping individuals within a specific environment survive long enough for the local selection regimes to have an

effect and make any necessary genetic adjustments (Via and Lande 1985). Regardless, the lack of GxE interactions does not mean there is an absence of local adaptation; the fact that outbreeding influenced development rate in both years not only confirmed that midhatch timing has a genetic basis, it also suggests that it is locally adapted in both broodlines.

Spatial hybrids in both broodlines showed signs of OBD, even though they were three generations removed from the initial cross, and hybridization affected developmental rate the same way in each run: the introgression of Pillar Creek genes into the Auke Creek genome prolonged development. Hybrid embryos, whose genome was composed of Pillar Creek genes (50%) and Auke Creek genes (50% + Auke Creek maternal influences), developed more slowly than controls which had only Auke Creek genes. The magnitude of this response in each broodline, however, differed. Midhatch was delayed approximately one week (+42 ATUs) in the even-year broodline, whereas it was only delayed 2.5 days (+12 ATU) in the odd-year counterpart. This discrepancy may be due, at least in part, to genetic differences that exist between broodlines and locations. Although some of the difference in development rate may have been due to differences in incubation temperature regimes, the thermal environment was likely not the major cause of this dissimilarity since the average daily water temperature was similar between years. Genomic differences also meant that OBD influenced inheritance patterns differently in each broodline, but the mechanism of the disruption was likely the same: spatial hybridization probably altered the proportions of additive variation, possibly by influencing the canalization process.

Because our breeding experiment did not include any previous generations of spatial hybrids, we could not determine which model of OBD (additive vs. non-additive) explained the observed disruption of midhatch timing. Wang et al. (2007), however, did compare midhatch times of the F_1 and F_2 hybrids to those of controls and backcrosses, and found that neither model alone adequately explained any of the observed differences.

Although the distance separating the source populations used in this study to create spatial hybrids exceeded what is likely to occur in nature or a fishery enhancement program, such hybridization did serve to document the occurrence of OBD in pink salmon, indicate that the introgression of nonnative fish may erode fitness by altering locally adapted traits, and demonstrate that its influences can last at least three generations. It is obvious, however, that additional information is needed. Our understanding of the mechanisms that drive OBD are still vague and we are still unsure exactly how long its effects can last. We are also not certain what geographic distances and habitat parameters are accurate predictors of genetic divergence and, therefore, OBD. Until these questions are addressed, salmon stocks should not be treated as homogeneous populations and caution must be taken when making management decisions that could result in outbreeding within or between wild and cultured populations if we are to maintain salmon fitness and productivity.

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Table 1.1. Genetic components estimated from the phenotypic variation among the offspring produced from a 2x2 factorial breeding design (Lynch and Walsh 1998); V_A is the additive genetic variance, V_D is the dominance genetic variance, V_{AA} is the variance associated with additive x additive epistasis, V_{AD} is the variability deriving from additive x dominance epistasis, V_{DD} is the variability from dominance x dominance epistasis, and V_M is the maternal effects variance that derived from maternally inherited genetic effects as well as common maternal environmental influences. High order effects include sources of genetic variation deriving from epistatic interactions that involve three or more loci. The asterisk indicates interaction.

Observed Phenotypic Variance	Genetic and Environmental Interpretation
Sire (σ_S^2)	$^1/_4V_A + {}^1/_{16}V_{AA}$ + Higher Order Effects
Dam (σ_D^2)	$^1/_4V_A + {}^1/_{16}V_{AA} + V_M$ + Higher Order Effects
Dam * Sire (σ_{DS}^2)	$^1/_4V_D + {}^1/_8V_{AA} + {}^1/_8V_{AD} + {}^1/_{16}V_{DD}$ + Higher Order Effects

Table 1.2. Significance of tests (REML) for the effects of dam, sire, family, incubation temperature, and hybridization on the development rate of even- and odd-year broodlines of native Auke Creek pink salmon and their spatial hybrids. The compartment effect evaluated the influence of a family's location within an incubation tray on development rate. Development rate was measured by accumulated thermal units (ATU) and the number of days required to reach mid-hatch. Grey shading indicates significant effects ($p \leq 0.10$).

	BROOD YEAR 2000		BROOD YEAR 2001	
	ATU	Days	ATU	Days
Native Auke Creek Fry				
Sire	0.672	0.655	0.026	0.027
Dam	0.825	0.808	0.022	0.022
Dam * Sire	0.212	0.219	0.798	0.782
Compartment	<0.001	<0.001	<0.001	<0.001
Temperature Effects				
Temp	<0.001	<0.001	<0.001	<0.001
Sire	0.051	0.036	0.113	0.096
Dam	0.072	0.079	0.070	0.021
Dam * Sire	0.684	0.382	0.334	0.249
Temp * Sire	0.153	0.480	0.179	0.113
Temp * Dam	0.264	0.624	0.022	0.098
Temp * Dam * Sire	0.215	0.432	0.787	0.789
Compartment	<0.001	<0.001	<0.001	<0.001
Hybridization Effects				
Cross	<0.001	<0.001	<0.001	<0.001
Sire	0.052	0.052	0.115	0.116
Dam	0.073	0.073	0.085	0.086
Dam * Sire	0.209	0.209	0.208	0.208
Compartment	<0.001	<0.001	<0.001	<0.001

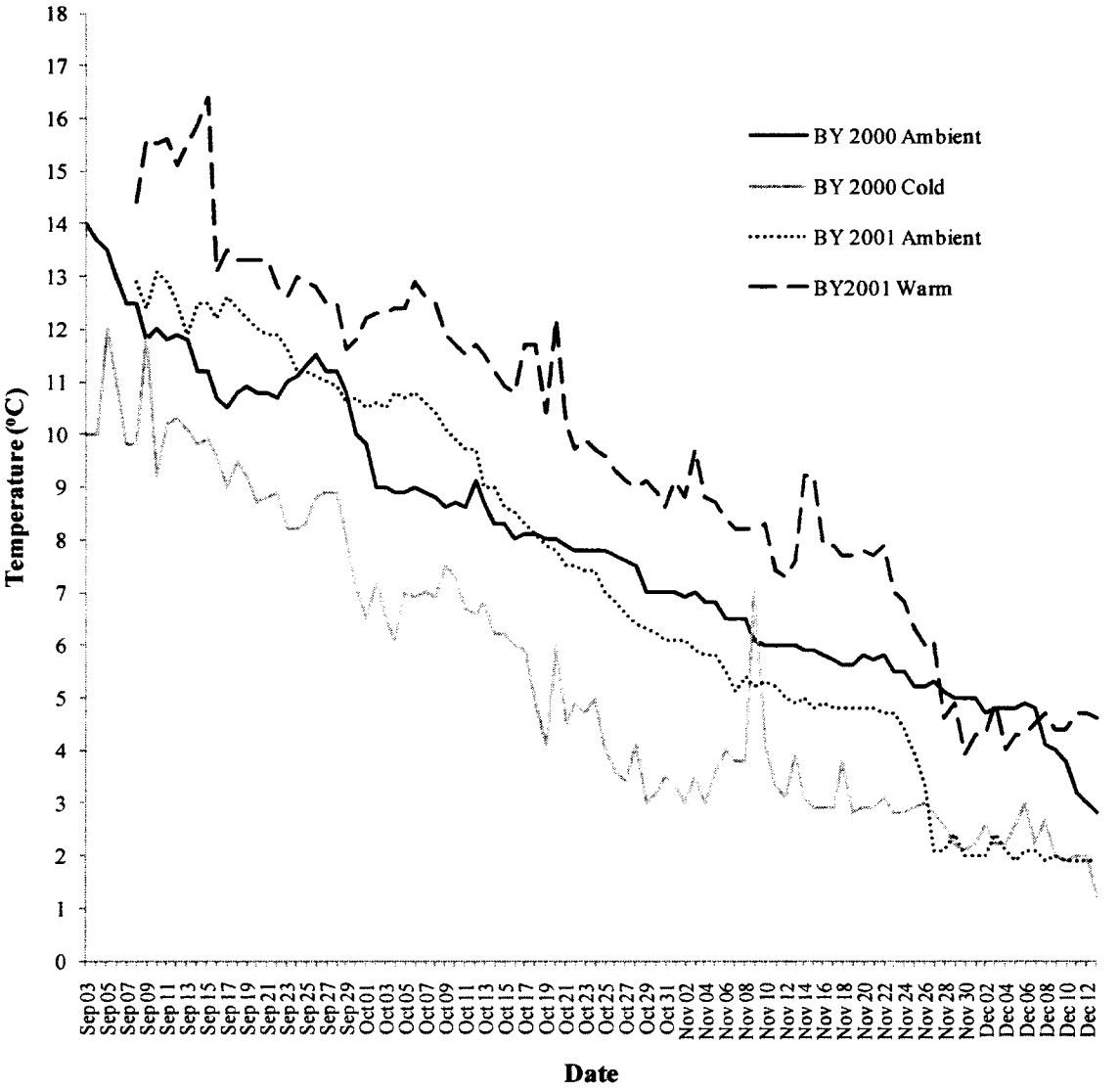

Figure 1.1. Water temperatures of rearing environments for brood year 2000 and 2001 pink salmon reared at Auke Creek Hatchery near Juneau, Alaska. Ambient temperature represents Auke Creek stream temperature for a given brood year, whereas the cold and warm regimes were designed to mimic the natural variation of cooler and warmer incubation environments.

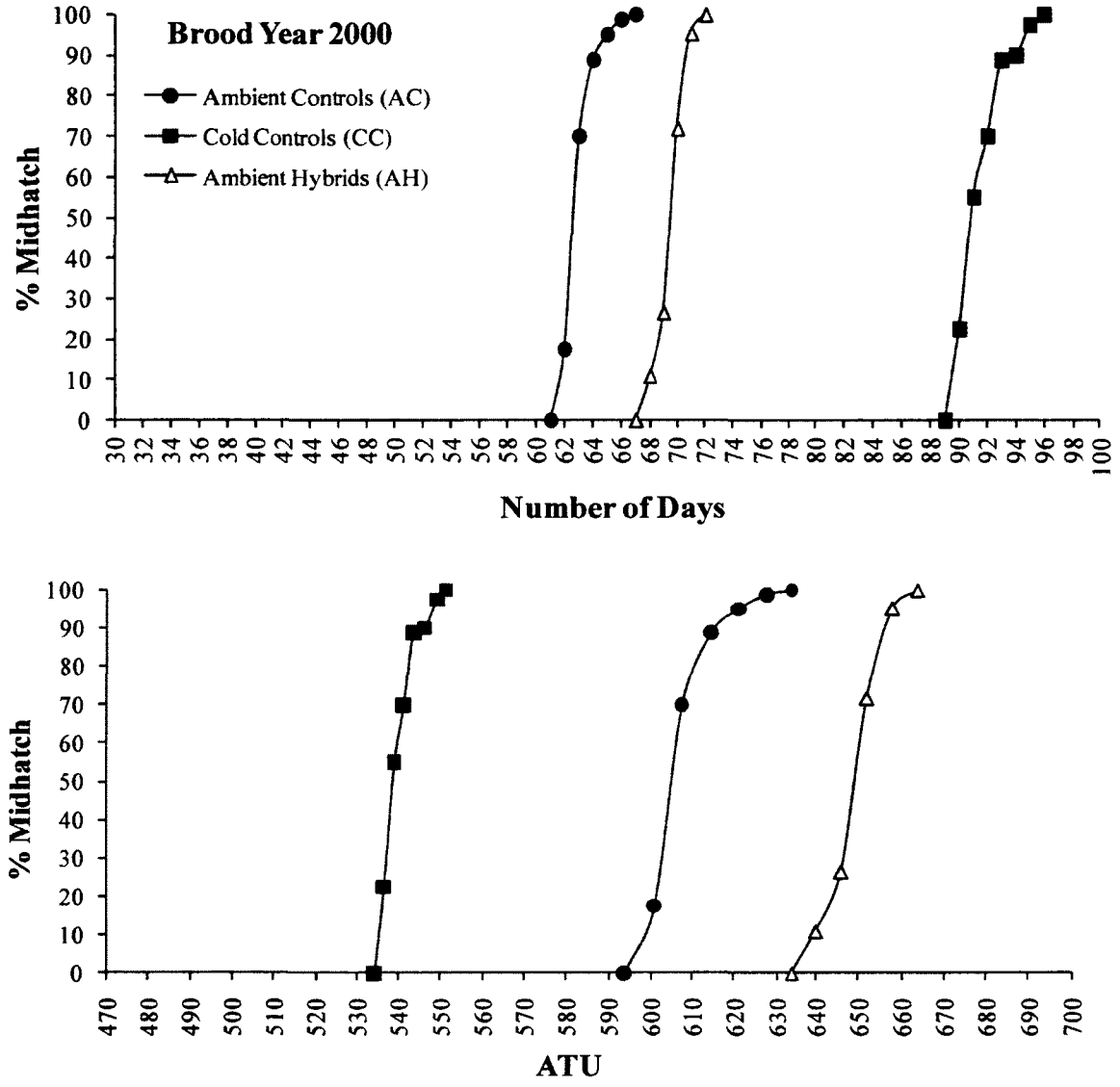

Figure 1.2. Cumulative percentage of pink salmon families reared at Auke Creek Hatchery, Juneau, Alaska to reach midhatch by total number of days and accumulated thermal units (ATUs) during brood year 2000. Families consisted of native pink salmon reared in ambient (AC) and cold waters (CC), as well as F_3 spatial hybrids incubated in ambient waters (AH).

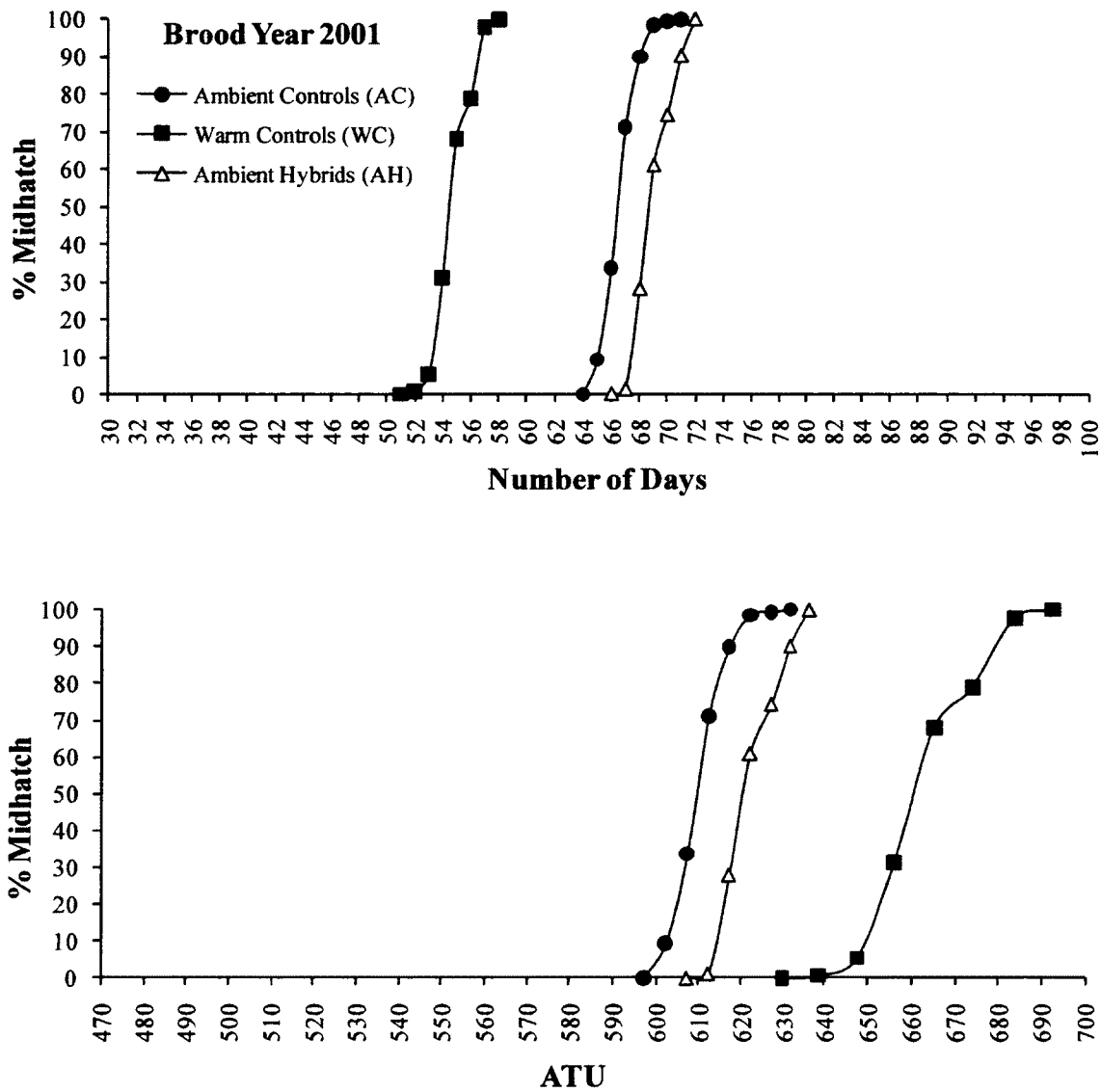

Figure 1.3. Cumulative percentage of pink salmon families reared at Auke Creek Hatchery, Juneau, Alaska to reach midhatch by total number of days and accumulated thermal units (ATUs) during brood year 2001. Families consisted of native pink salmon reared in ambient (AC) and warm waters (WC), as well as F_3 spatial hybrids incubated in ambient waters (AH).

CHAPTER 2

GENETIC AND ENVIRONMENTAL EFFECTS ON OTOLITH FORMATION[1]

[1] Oxman, D.S., Smoker, W.W., Hagen, P., and Gharrett, A.J. In preparation. Genetic and environmental effects on otolith formation. Canadian Journal of Fisheries and Aquatic Sciences.

ABSTRACT

Full- and half-sibling families of pink salmon from Auke Creek, Alaska and of outbred hybrids between Auke Creek females and Pillar Creek males from Kodiak Island, Alaska (1000 km distant) were incubated in ambient to determine how inheritance and hybridization influenced otolith formation. Native Auke Creek families were also incubated in chilled water to determine how environment affected otolith formation. The results indicated that an additive genetic effect played a role in early otolith development, but the phenotypic expression of these genes is plastic and strongly influenced by the environment. Lower incubation temperatures significantly affected all aspects of otolith morphology by slowing growth. Hybridization had little effect on otolith morphology, although it altered the proportions of additive and environmental variation, possibly by influencing the canalization process. The occurrence of crystallized otoliths was correlated with low incubation temperatures, but no genetic component was associated with their formation. Otolith shape was strongly canalized and was not affected by sire or dam effects, interactions between dam and sire, microhabitats, or spatial hybridization. Because genotype-by-environment effects were not observed in otolith development, we concluded that locally adapted genes were not associated with otolith formation.

Keywords: salmon, otolith, developmental variation, inheritance, local adaptation

INTRODUCTION

Otoliths are biologically inert, paired calcareous structures located in the inner ear of teleost fish. Each individual possesses three pairs of otoliths, the largest of which are the sagittae that grow incrementally and continuously throughout the life of an individual. The size, shape, ring patterns, and chemical composition of sagittal otoliths can vary substantially within a species; and this variation has been used to identify stocks (Bergenius et al. 2006), reconstruct life histories (Campana and Casselman 1993), and separate stocks in mixed stock fisheries (Begg et al. 2001)

Intraspecific variation in otolith morphology exists because its development is sensitive to environmental and biological change. The ectothermic nature of fish metabolism makes otoliths susceptible to environmental change, particularly water chemistry and temperature (Savoy and Grecco 1987, Radkte and Shafer 1992, Bestgen and Bundy 1998). Their formation can also be affected by the physiological changes associated with hormonal cycles and aging (Beckman and Wilson 1995, Campana 1999). All these factors have at least one thing in common: they are associated with metabolic processes, either directly or indirectly.

Because metabolic processes are regulated by genes, some degree of genetic control over otolith development is expected. Little, however, is known about the mechanisms of this control. Both paternal and maternal genomes can influence otolith formation (Høie et al. 1999, Yamamoto and Reinhardt 2003), but the nature of this control and the genetic sources of phenotypic variation remain unknown. Tracing

variation in otolith structure to its underlying genetic sources can be difficult since the genetic signal can be confounded by environmental influences, so the question often remains: does intraspecific variation in otolith structure occur because genotypic expression is affected by environmental conditions (phenotypic plasticity), or is it the result of additive, epistatic and/or dominance interactions among genes? These scenarios are not mutually exclusive. It is also possible that intraspecific variation results in local adaptation.

In philopatric species that have numerous local populations across a diverse range of environments, there is a potential for genomes of the populations to become adapted to local conditions. The well documented homing ability of salmon to their natal streams for spawning promotes reproductive isolation, thereby increasing the potential for such adaptation as genes and gene complexes become adapted to local conditions (Taylor 1991). To determine if a trait is locally adapted, it must be demonstrated that variation in the character under consideration has a genetic basis and that it enhances survival and/or reproduction for a deme while it is in its particular environment (Kawecki and Ebert 2004). One would therefore expect to see local differences in fitness-related traits such as growth rate, size, fecundity, homing ability, and temperature tolerance (Taylor 2004). Such evidence, however, is difficult to obtain. Instead, genotype-by-environment (GxE) interactions have been used to infer that a trait may be locally adapted (Via and Lande 1987, Lynch and Walsh 1998). Genotype-by-environment interactions occur when different genotypes respond to the same environmental change in different ways. These interactions have been associated with a variety of fitness-related traits in salmon

including survival (Evans et al. 2010), development rate (Hebert et al. 1998), and fin size (Taylor and McPhail 1985), but have never been associated with otolith formation despite the fact their development is sensitive to environmental variation.

Our primary objective was to determine how parental genomes influence otolith development in pink salmon (*Oncorhynchus gorbuscha*). We also asked whether exposure to environmental change early in development and hybridization affected otolith formation to provide evidence of GxE effects (local adaptation) and disrupted canalization. Several steps were taken to accomplish these objectives: (1) full- and half-sib families of Auke Creek (Juneau, Alaska) pink salmon embryos were incubated in ambient Auke Creek water to provide information about the parental effects on otolith formation; (2) some of these families were also reared in chilled water for the first 14 weeks to determine how changes in the thermal environment early in development influenced genotypic expression (GxE effects); and (3) third generation (F_3) hybrids between spatially separated populations (herein referred to as "spatial hybrids") were bred to see if outbreeding altered otolith development.

MATERIALS AND METHODS

Breeding and Incubation

Pink salmon were collected at Auke Creek Research Station (a facility of US National Oceanic and Atmospheric Administration's Alaska Fisheries Science Center)

from a weir near the mouth of Auke Creek, a 350 m high-gradient, lake-fed stream located near Juneau, Alaska, a stream in which populations of pink salmon naturally spawn. Mature salmon used in this experiment were fin-marked individuals that represented the second filial (F_2) generation of a breeding experiment involving native Auke Creek pink salmon and spatial hybrids that had been released as fry to the North Pacific Ocean from the Auke Creek Research Station during the spring of 1999. The hybrid lineage was originally created by crossing late-returning females in Auke Creek with late-returning males from Pillar Creek on Kodiak Island, Alaska (Gilk et al. 2004). Pillar Creek is a 1800 m long reservoir-fed stream located about 1000 km west of Juneau that is historically 1 to 2 °C cooler than Auke Creek between late August when the salmon spawn and mid-November when their eggs hatch. Although the latitudes (near 58°N) and habitats of Auke and Pillar Creeks are similar, their spatial separation and different temperature regimes make it likely that the two runs evolved independently into genetically distinct and locally adapted populations (Adkison 1995, Gilk et al. 2004). Consequently, crosses between these demes produced spatial hybrids that allowed us to study the effect of outbreeding on otolith development.

In late August 2000, the F_2 generation of maturing salmon, descendants of the natives and hybrids bred in 1996 by Gilk et al. (2004) and in 1998 by Wang et al. (2007), were collected as they returned to Auke Creek, sorted according to gender and fin clip (native or hybrid), and held for several days until maturation was complete. On 3 September 2000, a blocked 2x2 factorial design was used to produce full- and half-sib F_3 families from native (20 males and 20 females) and hybrid (16 males and 16 females)

returning fish. There were 10 blocks of natives (40 families) and 8 blocks of hybrids (32 families).

Fertilized eggs were incubated in divided trays (FAL™; Marisource Milton, WA) that housed each family separately. Each tray had 10 compartments which contained single families. These trays were placed in incubation cabinets so that each cabinet contained eggs from a single type of cross (native or hybrid). The eggs in one native and one hybrid cabinet were incubated in ambient temperature Auke Creek water. Because water temperatures in the stream, intragravel environment (redds), and research station are similar, development of natives reared under ambient conditions represented the normal development of wild Auke Creek pink salmon. These natives were the control group. A subset of eggs from the native families were placed in third cabinet and incubated in a simulated environment that was, on average, 2.4 °C (± 0.9 °C) cooler per day than ambient Auke Creek stream temperatures through hatching (Fig. 2.1). All families were divided into four approximately equal portions, two of which were randomly assigned to compartments within a cabinet in each temperature regime to provide replication.

The temperature in the simulated environment was altered daily to mimic natural variation in incubation conditions (Fig. 2.1). Water in the cold regime was re-circulated to achieve the level of control needed to simulate daily thermal fluctuations in a captive setting. Two 1,136-liter Living Streams® (Frigid Units, Inc. Toledo, OH) were used as a reservoir and equipped with a 1-hp chiller. A ¾-hp submersible pump was used to move water from the reservoir to a 76-liter head tank, which distributed water to the incubation

cabinets by a gravity feed. Water from the incubators drained into the reservoir, where it was conditioned and pumped back to the head tank. Water in the reservoirs was continually replaced with fresh stream water; a complete turnover occurred every 5 hours. The experiment was designed to determine if exposure to thermal stress early in development affected otoliths. Consequently, the water was not chilled after the 14th week of development when ambient temperature Auke Creek water was allowed to flow unidirectionally and water temperatures in the two regimes were identical (Fig. 2.1).

Water was supplied to all incubators at a rate of 8 L/min until the eyed stage was reached and at 23 L/min thereafter. Temperatures were recorded daily to the nearest 0.1 ^{o}C and did not differ substantially between incubation cabinets for a particular temperature regime. Before hatching, incubating eggs were treated once a week with formalin (1:6000 in static water) for one hour to reduce infestations by fungus and algae, a standard practice in the culture of salmon embryos (Wood 1968). After hatching, weekly salt treatments (3 parts per thousand sodium chloride for 1 hour) were used to minimize infestations, another common practice in salmon culture. All water was filtered through sheer nylon mesh prior to entry into the incubation cabinets to prevent hydra infestation.

Otolith Measurements

Fry were collected after yolk absorption from all families. By sampling fry after yolk consumption was complete, we ensured that development was standardized across

treatment groups and controlled for age-related bias. Six siblings per replicate (12 fry per family) were sampled from each cross and temperature regime. The standard length (SL) for each individual was recorded. This sampling scheme produced three treatment groups: ambient native controls (AC), cold natives (CC), and ambient hybrids (AH).

Sagittal otoliths were removed from each fry, mounted sulcus-side up on glass slides in thermoplastic cement, and photographed at 25X magnification with a hi-resolution 5 megapixel (2592 x 1944 pixels) Leica DFC420 digital camera. The system was calibrated at 25X magnification with a slide micrometer. Measurements were recorded to the nearest micron. Optimas 6.5 Image analysis software was used to quantify six physical characteristics for each left and right otolith: area, perimeter, longest length, breadth, maximum radius, and minimum radius. Radii measurements originated from the otolith's center of mass, which was located in the central-most region of the otolith where multiple primordia fused to form the otolith's core. Because the longest length transect seldom passed through the center of mass, radii measurements did not coincide with the longest length transect. A rectangularity index, defined as the ratio of otolith area to the area of an enclosing bounding box oriented along the otolith's major axis (Optimas 1996), described otolith shape. An index of 0.5 indicated that the boundary box was square. The index increases as the boundary box becomes more rectangular and an index of 1.0 is a line. Lastly, the crystalline composition of each otolith was noted because the calcium carbonate in sagittae can be deposited in one of two forms; it typically occurs as a milky-white form called aragonite but it can also be deposited in a clear, crystallized

polymorph known as vaterite. An aragonitic otolith was assigned a dummy variable value of "0" and the crystallized vaterite form was assigned a "1".

Statistical Analysis

Restricted maximum likelihood analysis (REML) was used to determine how parents, incubation temperature, and spatial hybridization influenced the variation associated with the development of sagittal otoliths. Restricted maximum likelihood is more robust to deviations from normality, non-homogenous variances, and unbalanced designs in mixed-model comparisons than ANOVA (Lynch and Walsh 1998, Van Dongen et al. 1999). The REML method uses the Z-statistic, defined as the estimated covariance parameter divided by its approximate standard error, to test random effects for significance. The *F*-statistic is used to evaluate fixed effects. Otolith measurements and morphological status were compared among families within a treatment with the model:

$$(1) \quad Y_{klmno} = \mu + B_k + D_{kl} + S_{km} + D_{kl}{*}S_{km} + R_{klmn} + \varepsilon_{klmno}$$

where Y_{klmno} was the dependent variable (otolith length, area, etc.), μ was the population mean, B_k was the block effect (i.e. the independence and randomness of the experimental design), D_{kl} was the dam effect, S_{km} was the sire effect, $D_{kl}{*}S_{km}$ was the interaction between dam and sire (i.e. family effects), R_{klmn} was the effect of compartment position on development (i.e. microhabitat effects), and ε_{klmno} was the residual random error (i.e. variation within families). All factors in this model were random.

Another model was used to test the effects of temperature on otolith size and shape:

$$(2) \quad Y_{jklmno} = \mu + T_j + B_k + T_j*B_k + D_{kl} + S_{km} + D_{kl}*S_{km} + T_j*D_{kl} + T_j*S_{km} + T_j*D_{kl}*S_{km} + R_{jklmn} + \varepsilon_{jklmno}$$

where T_j was the effect of incubation temperature, T_j*B_k was the interaction between temperature and block (i.e. the effect of temperature on the randomness and independence of the experimental design), T_j*D_{kl} was the effect of the interaction between temperature and dam (i.e. environmental interactions related to dam effects), T_j*S_{km} was the effect of the interaction between temperature and sire (i.e. GxE interactions related to sire effects), and $T_j*D_{kl}*S_{km}$ was the interaction between temperature and the family effect (i.e. GxE interactions related to family effects).

The model used to determine if spatial hybridization influenced otolith development was:

$$(3) \quad Y_{iklmno} = \mu + C_i + B_{ik} + D_{ikl} + S_{ikm} + D_{ikl}*S_{ikm} + R_{iklmn} + \varepsilon_{iklmno}$$

where C_i was the effect of cross. Equations 2 and 3 were mixed-models because they contained both random (block, dam, sire, replicates) and fixed (cross, temperature) effects.

Another potential result of developmental instability that may arise from either genetic or thermal stress is increased phenotypic variation (Palmer and Strobeck 2003). Therefore, the variation associated with each otolith measurement was also compared among treatments with Levene's test for homogeneous variances to determine if

treatment increased phenotypic variability, which would be considered an indicator of disrupted development.

Because the relationships within and among families were known, we used a quantitative genetics approach to express the variability partitioned by each model in terms of its underlying causal genetic components (Lynch and Walsh 1998). Given our breeding design, the variation in otolith size and shape associated with sire effects would imply that the heritable source of such variability came mostly from additive genetic factors (Table 2.1). Dam-associated variability also would primarily provide evidence of an additive effect and maternally inherited effects such as common maternal environmental influences, which include egg quality and egg size (Table 2.1). Interactions between dam and sire would imply that variation in otolith morphology that came from non-additive genetic effects such as dominance and/or epistatic effects (Table 2.1). Therefore, by using REML to test for significant dam, sire, and family effects, we evaluated the underlying genetic components of variation on otolith development.

Our experimental design also allowed us to determine if otolith traits were potentially influenced by locally adapted genes. By rearing members of each native family in two thermal environments, we were able to use model 2 to determine if GxE interactions occurred between dam and temperature, sire and temperature, and family and temperature. Significant GxE interactions for a given trait would mean that different genotypes (different families) respond to environmental change in different ways, and such responses indicated that trait had the potential to be locally adapted (Lynch and Walsh 1998). The absence of GxE interactions would indicate that genotypes responded

similarly to environmental change and that any observed differences in otolith morphology were probably caused by phenotypic plasticity rather than genetic differences. Because GxE effects associated with dams are confounded by common environmental effects such as egg quality and egg size, only GxE effects associated with sires and families were used to evaluate otolith traits for local adaptation.

All comparisons and hypothesis tests were conducted in SAS 9.1with PROC MIXED (SAS Institute, 2002). The significance level used for hypothesis testing was $p \leq 0.10$. Native fry reared at ambient temperature (controls) were used to establish the normal range of otolith size, shape, and variability of the reference population (i.e. Auke Creek pink salmon). Differences in otolith size or shape among native fry incubated in cold water relative to that of natives raised at ambient temperature was presumed to indicate that developmental processes were influenced thermally. Similarly, differences observed in the size or shape of otoliths from spatial hybrids relative to that of controls was presumed to reflect the genetic differences between hybrids and natives and was considered indicative of outbreeding.

RESULTS

Four hundred and eighty pairs of otoliths were recovered from both AC and CC fry, and 384 pairs were collected from AH fry. Because correlations between the otolith measurements and fish length (SL) were low for both left and right otoliths ($R^2 < 0.55$) and fry length did not differ among treatments (≤ 0.4 mm; $p \geq 0.61$), otolith

measurements were not standardized by SL. Similarities in SL among treatments also meant that differences in otolith structure and underlying genetic influences among groups could not be attributed to differences in fish length or developmental stage.

Native Auke Creek Fry (Controls)

Sagittal otoliths from AC fry provided reference information for otolith size and shape for native Auke Creek pink salmon. The length of left otoliths averaged 593 ± 30 μm, breadth was 454 ± 21 μm, minimum radius was 204 ± 10 μm, and maximum radius was 315 ± 18 μm. Perimeter was 1,749 ± 82 μm and area was 196,221 ± 15,826 μm^2. Measurements of right otoliths were similar to those of their left counterparts; each trait differed by less than 0.38% (Fig. 2.2). These dimensions resulted in left and right otoliths that had a rectangularity index of 0.73 (± 0.02). Approximately 5.6% and 8.1% of left and right otoliths, respectively, were crystallized but the difference was not significant (p = 0.13; Fig. 2.2).

Comparisons among AC families indicated that the area, length, maximum radius, and perimeter of left otoliths were influenced by both dam and sire effects (Table 2.2), which implied that additive genetic factors played an important role in the expression of these attributes. Although dams had a similar effect on right otoliths, the sire effect influenced that side differently, influencing only the area and breadth (Table 2.2). Interactions between dam and sire did not significantly influence the morphology of either left or right otoliths (Table 2.2), indicating that non-additive variation (e.g.,

dominance and epistatic effects) had little effect on variation in early otolith development. Neither dam, sire, nor interactions between dam and sire affected the variation in overall shape or crystalline composition of left and right otoliths (Table 2.2).

A family's position within an incubation tray influenced some aspects of otolith development. Microhabitat altered the area, breadth, and minimum radius of both left and right otoliths, as well as the perimeter of right otoliths (Table 2.2). These attributes were 0.95% to 4.40% larger than average in families located in the front left-side of a tray furthest from the water source, whereas they were 1.85% to 5.24% smaller than average in families located in the same position on the right side of the tray. The position of other compartments within a tray had little effect on otolith size; measurements of traits from these compartments differed from their overall means by 0.04% or less. Incubation position did not change the shape or crystalline composition of left or right otoliths (Table 2.2).

Temperature Effects

Otoliths from fry reared in cold water were compared to those of fry raised at ambient temperatures to determine if the thermal environment influenced otolith development and morphology. Cold incubation temperature reduced otolith development significantly; all aspects of left and right otoliths from CC fry were 2.56% to 5.00% smaller than those of AC fry (Fig. 2.2). The effect was greater for area, which was reduced by approximately 10% (Fig. 2.2). Otoliths from CC fry were also less rectangular

and more square than their ambient counterparts (Fig. 2.2). All differences between treatments were significant (Table 2.2).

The presence of crystallized otoliths was correlated with incubation temperature (Table 2.2). Left otoliths from fry reared in cold water were 4 times more likely to be vateritic than those from the left side of fry reared in ambient water (Fig. 2.2). Similarly, right otoliths from fry incubated in cold water were 3 times more likely to be crystallized than those from fry reared at ambient stream temperatures (Fig. 2.2).

The variability associated with each otolith trait did not differ between incubation environments in either left or right otoliths ($p \geq 0.110$), with the exception of area, which was less variable in both CC fry otoliths ($p \leq 0.002$; Fig. 2.2).

Comparisons of traits and indices from left and right otoliths among families and temperature regimes using REML indicated that no GxE effects were associated with dams, sires, or families (Table 2.2). It is therefore likely that the observed variation in morphology was the result of phenotypic plasticity. Changes to the sire and dam effects associated with fry reared in the cold environment relative to those observed among fry incubated at ambient temperatures, however, suggested that the cooler incubation temperature altered the proportion of additive genetic variation that was associated with otolith formation (Table 2.2).

Compartment location within the cold water incubators influenced the dimensions of both left and right otoliths (Table 2.2). All traits in both otoliths were reduced by 0.85% to 5.34% in families located in the front of the tray furthest from the water source, whereas otoliths from families reared in the middle of the tray were 0.72% to 3.37%

larger than average. The size of otoliths in the remaining compartments did not change; measurements of traits from these compartments differed from their overall means by 0.46% or less. Incubation position did not alter the shape or crystalline composition of left or right otoliths (Table 2.2).

Hybridization Effects

Otoliths from native pink salmon and their spatial hybrids incubated at ambient temperatures were compared to determine if outbreeding influenced otolith formation and inheritance mechanisms. All dimensions of the left and right hybrid otoliths were 0.5% to 2.0% larger than those of their native counterparts (Fig. 2.3). These changes, however, were only significant for length in left and right otoliths and maximum radius in right otoliths (Table 2.2).

Hybridization did not influence otolith shape or crystalline structure (Fig. 2.3). Rectangularity indices in AH fry were nearly identical to those of AC fry in both left and right otoliths (Table 2.2). Crystallized otoliths from each side were approximately 2.00% more frequent in hybrids than in their native counterparts (Fig. 2.3), but this difference was not significant for either otolith (Table 2.2).

Levene's test indicated most traits tended to be less variable in hybrid fry than in native fry: the length, breadth, area, and perimeter of left hybrid otoliths were less variable than those of native fry ($p \leq 0.011$), though the variability associated with minimum and maximum radius was equivalent between crosses (Fig. 2.3). All traits in

right otoliths, with the exception of minimum radius, were less variable in hybrid fry ($p \leq 0.051$; Fig. 2.3). The variability associated with the shape of left and right otoliths was similar between crosses ($p \geq 0.848$).

Some parental effects were affected by hybridization. In contrast to native sires, there was no significant sire effect on the development of left otoliths by hybrid sires (Table 2.2). Although hybrid sires did affect right otoliths, this influence differed from that of native sires by contributing to the variability of length and maximum radius (Table 2.2) instead of area and breadth as observed among native families. Interactions between dam and sire were not observed among native families, but they did occur in the length and maximum radius of right otoliths of hybrid fry (Table 2.2). Most components of inheritance associated with otolith formation in hybrids were similar to those observed among natives and were unaffected by outbreeding (Table 2.2); the hybrid dam effect influenced almost all aspects of left and right otoliths, interactions between hybrid parents had little impact of otolith development, and neither dam, sire, nor family effects influenced the overall shape or crystalline composition of left and right hybrid otoliths (Table 2.2).

Like the other treatments, compartment location within the incubators containing hybrid families altered the size of left and right otoliths (Table 2.2). Most attributes in each otolith were reduced 1.50% to 6.14% in families located at the front of an incubation tray furthest from the water source, whereas they were 1.43% to 5.46% larger than average in families incubated in the middle of the tray. The size of otoliths from fry in the remaining compartments was not affected and measurements of otolith traits from

these other compartments differed from their overall means by 0.14% or less. Incubation position did not change the shape of otoliths, but more crystalline otoliths were formed in families located furthest from the water source (Table 2.2).

DISCUSSION

There were several indications that both genetic and environmental factors influenced early otolith development in pink salmon. Observations from our breeding experiment indicated that early otolith formation was influenced primarily by additive genetic inheritance, and some dam effects indicated that maternal effects such as egg size and/or quality may also be important. The phenotypic expression of the genes that influenced otolith formation, however, was very sensitive to environmental factors.

Much of the variation observed in the otoliths was environmental; both variation in water temperature and microhabitats within incubation trays strongly influenced the otolith phenotype. Exposure of embryos to low incubation temperatures through hatching significantly slowed otolith formation, changed otolith shape, and was correlated with a high incidence of crystallized (vateritic) otoliths. The thermal disruption of developmental pathways was not unexpected because fish are ectothermic and, as such, their metabolic processes are regulated largely by their external environment. Indeed, changes in water temperature and chemistry have been correlated with changes in otolith morphology in many species (Volk et al. 1999, Tomas and Geffen 2003, Cardinale et al. 2004, Barnett-Johnson et al. 2008).

Given this sensitivity to environmental change, it is not surprising that a family's position within an incubation tray also influenced otolith morphology within each treatment. Partitions within a tray that kept each family isolated likely caused subtle localized differences in water flow, chemistry, and temperature. The phenotypic variation caused by the varying environment within the compartments, however, was not enough to obscure the variability caused by genetic effects and were uncorrelated with genetic effects because families were assigned randomly to compartments within an incubator.

Genetic alteration caused by spatial hybridization would be expected to generate phenotypic variability because genes play a significant role in the regulation of fish metabolism and development. Our results, however, were ambiguous. Spatial hybridization altered the proportions of additive genetic variation by changing how the sire effect influenced otolith development, but the additive genetic influence of dams was essentially unaffected. Genotypic alteration, however, did not affect otolith development substantially. This relative stability could have been the result of genetic similarities between stocks, the persistence of the maternal effects, genetic and developmental flexibility (plasticity) in the source populations, or canalization, though none of these possibilities are mutually exclusive.

Canalization tends to be associated with fitness-related traits (reviewed by Stearns et al. 1995, Gaillard and Yoccoz 2003); and because morphology is important to otolith function, which is essential to fish survival (Tomas and Geffen 2003, Panfili et al. 2005, Gagliano et al. 2008), it is not surprising that some aspects of the otolith phenotype would be developmentally stable. Otolith shape and crystalline structure seemed to be

particularly resilient to genetic variation since neither attribute was affected by spatial hybridization. It is also important to note that although temperature had a large impact on otolith structure by decreasing its dimensions by 2.6% to 10.0%, it altered the shape index by only 1%. Conservation of shape and composition is reinforced by the fact that neither dam, sire, nor interaction between parents affected otolith growth. In addition, genotype-by-environment effects were lacking for all otolith traits, indicating that all genotypes responded similarly to environmental change. This suggests that the variation in phenotype was due to phenotypic plasticity, that locally adapted genes did not substantially influence otolith development, and that the genes that govern otolith formation may be similar among stocks.

Adding to the uncertainty associated with genetic influences was the observation that phenotypic variability decreased as a result of spatial hybridization, even though such hybridization would have been expected to cause developmental instability. Improved developmental stability could be indicative of heterosis, which can produce positive phenotypic changes as a consequence of the increased genetic variability that results from hybridization. When viewed in this context, the presence of heterosis indicates genetic disruption.

Our results demonstrate that both genetic and environmental factors influence otolith morphology. While genetic sources influence otolith development directly, environmental effects apparently altered development rates, which in turn modified otolith size, shape, and morphology. The distinction between genetically and environmentally derived variation is especially important when intraspecific differences

in otolith structure is used to reconstruct life histories and identify stock structure, Few, however, have attempted to make this distinction (Høie et al. 1999, Cardinale et al. 2004). In our study, we observed that changes in pink salmon otolith structure related to environmental variation were larger, more obvious, and more variable than those related to genetic variability. The genotypic effect on phenotypic variation was relatively small and sensitivity to environmental change did not vary among genotypes, indicating that the genes that control otolith formation were conserved and that genetically-induced variation was small. Consequently, if the majority of phenotypic variation in otolith structure is caused by environmental influences, then stock structure based on such variation may be more an indication that groups of fish within a species were exposed to different environmental conditions than it is an indication of genetic differences. Although our results may not apply to all species, it indicates that intraspecific variation in otolith morphology should be compared among groups with caution when it is used to evaluate population dynamics.

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Table 2.1. Genetic components estimated from the phenotypic variation among offspring produced from a 2x2 factorial breeding design; V_A is the additive genetic variance, V_D is the dominance genetic variance, V_{AA} is the variance associated with additive x additive epistasis, V_{AD} is the variability deriving from additive x dominance epistasis, V_{DD} is the variability from dominance x dominance epistasis, and V_M is the maternal effects variance that derived from maternally inherited genetic effects as well as common maternal environmental influences. High order effects include sources of genetic variation deriving from epistatic interactions that involve three or more loci. The asterisk indicates interaction.

Observed Phenotypic Variance	Genetic and Environmental Interpretation
Sire (σ_S^2)	$^1/_4V_A + {}^1/_{16}V_{AA}$ + Higher Order Effects
Dam (σ_D^2)	$^1/_4V_A + {}^1/_{16}V_{AA} + V_M$ + Higher Order Effects
Dam * Sire (σ_{DS}^2)	$^1/_4V_D + {}^1/_8V_{AA} + {}^1/_8V_{AD} + {}^1/_{16}V_{DD}$ + Higher Order Effects

Table 2.2. Probability values from REML models testing for the effects of dam, sire, family, incubation temperature, and hybridization on the dimensions of otoliths from native Auke Creek pink salmon and their spatial hybrids. The compartment effect evaluated the influence of a family's location within an incubation tray on otolith development. "Morph Status" refers to the crystalline composition of an otolith (aragonite vs. vaterite) and "Rect." refers to the rectangularity shape index. Grey boxes indicate significant effects ($p \leq 0.10$).

	LEFT OTOLITHS								RIGHT OTOLITHS							
	Area	Breadth	Longest Length	Maximum Radii	Minimum Radii	Morph Status	Perimeter	Rect.	Area	Breadth	Longest Length	Maximum Radii	Minimum Radii	Morph Status	Perimeter	Rect.
Native Auke Creek																
Sire	0.058	0.112	0.072	0.086	0.185	0.332	0.054	0.133	0.090	0.096	0.153	0.175	0.188	0.527	0.127	0.732
Dam	0.051	0.210	0.048	0.058	0.218	0.400	0.049	0.106	0.074	0.198	0.062	0.069	0.275	0.878	0.084	0.228
Dam * Sire	0.595	0.221	0.517	0.444	0.406	1.000	0.806	0.670	0.357	0.296	0.264	0.301	0.273	0.834	0.269	0.552
Compartment	0.030	0.012	0.580	0.586	0.027	0.382	0.131	0.736	0.013	0.011	0.198	0.112	0.031	1.000	0.029	0.317
Temperature Effects																
Temp	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.003
Sire	0.072	0.051	0.184	0.264	0.055	0.439	0.106	0.893	0.211	0.088	0.337	0.338	0.080	0.093	0.201	0.680
Dam	0.054	0.059	0.072	0.085	0.075	0.178	0.059	0.137	0.061	0.105	0.077	0.094	0.161	0.063	0.064	0.381
Dam*Sire	0.888	0.566	0.282	0.167	0.707	0.584	0.494	0.495	1.000	0.733	0.575	0.402	0.893	0.310	0.533	0.867
Temp * Sire	0.716	0.673	0.711	0.431	0.594	0.109	0.558	0.985	0.321	0.769	0.428	0.530	0.615	0.340	0.816	0.818
Temp * Dam	0.719	0.877	1.000	0.837	0.619	0.178	0.536	0.785	0.553	0.542	0.393	0.329	0.343	0.403	0.880	0.469
Temp * Dam * Sire	0.475	0.184	0.633	0.902	0.372	0.151	0.393	0.885	0.436	0.409	0.427	0.582	0.419	0.704	0.423	0.617
Compartment	0.005	0.075	0.048	0.120	0.079	0.778	0.007	0.250	0.011	0.027	0.055	0.064	0.086	0.612	0.021	0.479
Hybridization Effects																
Cross	0.283	0.668	0.093	0.128	0.337	0.309	0.428	0.773	0.307	0.788	0.077	0.055	0.379	0.544	0.311	0.226
Sire	0.778	0.912	0.126	0.108	0.426	0.912	0.956	0.354	0.585	0.714	0.078	0.065	0.410	0.294	0.247	0.177
Dam	0.079	0.131	0.075	0.076	0.087	0.621	0.065	0.124	0.099	0.207	0.065	0.058	0.122	0.232	0.067	0.278
Dam * Sire	0.655	0.912	0.723	0.709	0.460	0.891	0.695	0.621	0.466	0.717	0.085	0.021	0.545	0.553	0.148	0.833
Compartment	0.006	0.004	0.699	0.417	0.004	0.046	0.104	0.791	0.002	0.001	0.078	0.249	0.002	0.057	0.019	0.657

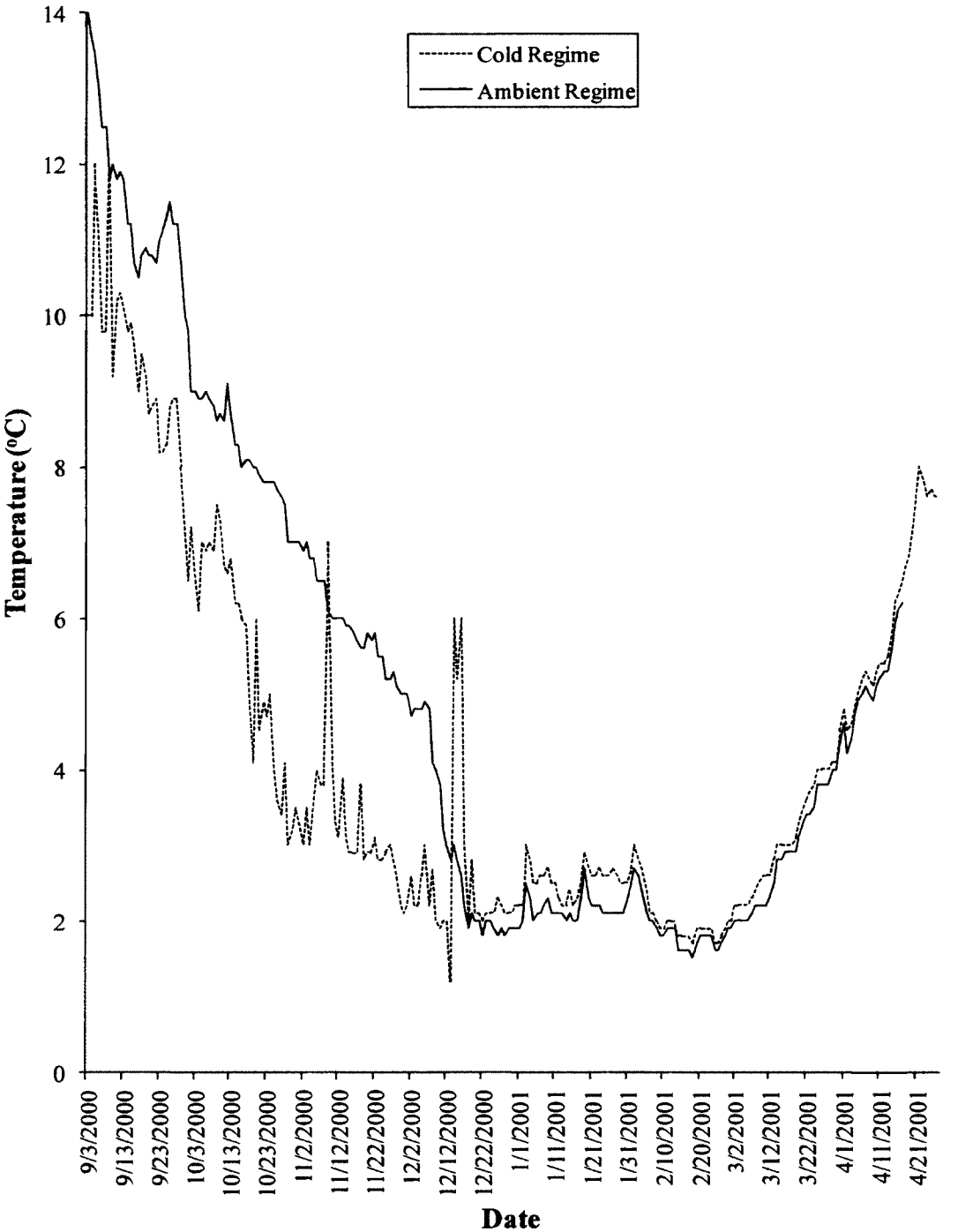

Figure 2.1. Water temperatures for two rearing environments for brood year 2000 pink salmon reared at Auke Creek Hatchery near Juneau, Alaska from 9/3/00 through 4/27/01. Ambient temperatures represent Auke Creek stream temperatures, whereas the cold regime was designed to mimic the natural variation of a cooler incubation environment. Water chillers were taken offline on 12/13/00.

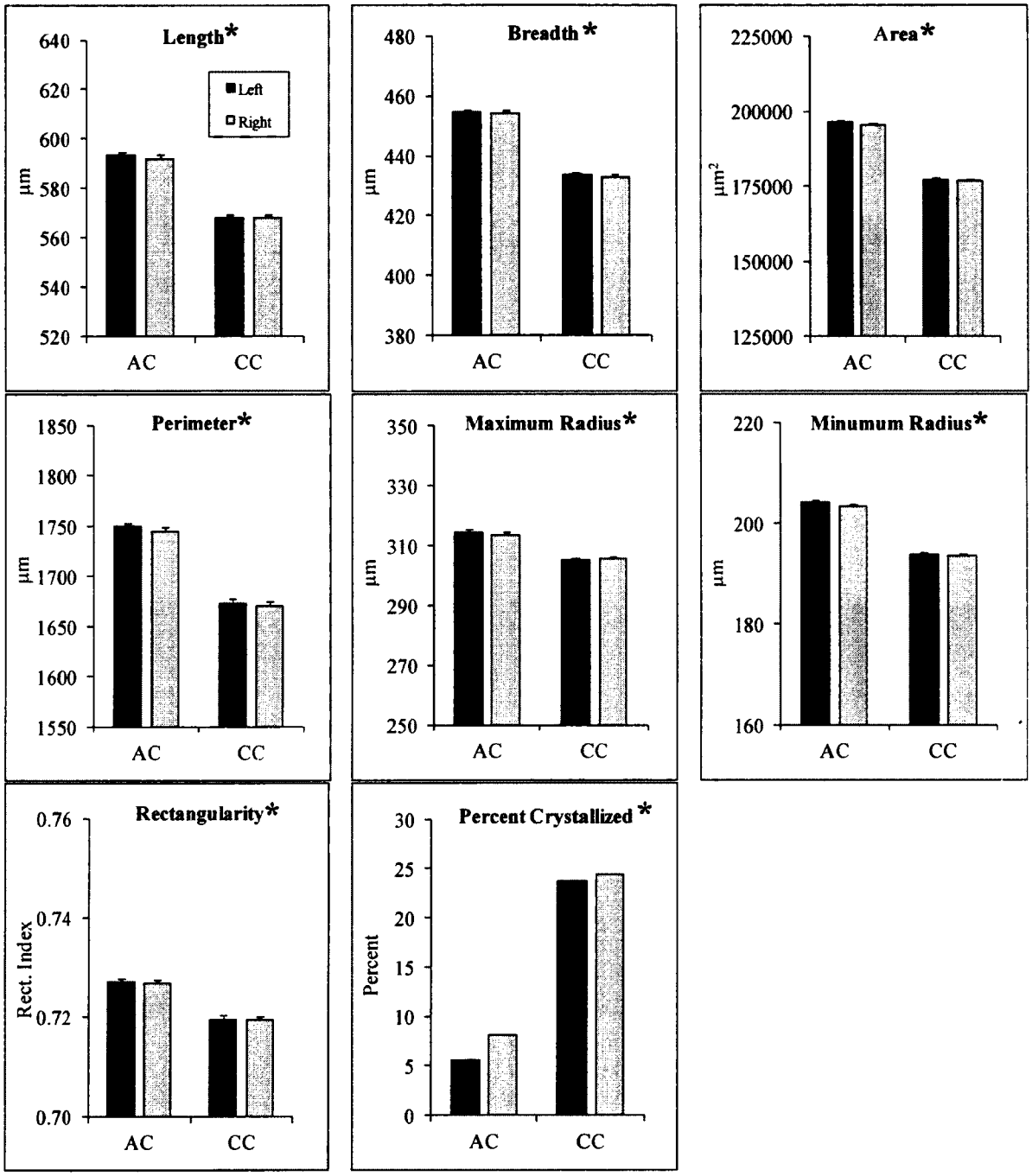

Figure 2.2. Measurements made on left (black) and right (grey) sagittal otoliths recovered from native pink salmon reared in ambient (AC) and cold waters (CC), as well as the percentage of otoliths that were vateritic (crystallized). Error bars represent standard error. The (*) indicates significant differences between incubation environments for both left and right otoliths ($p \leq 0.01$).

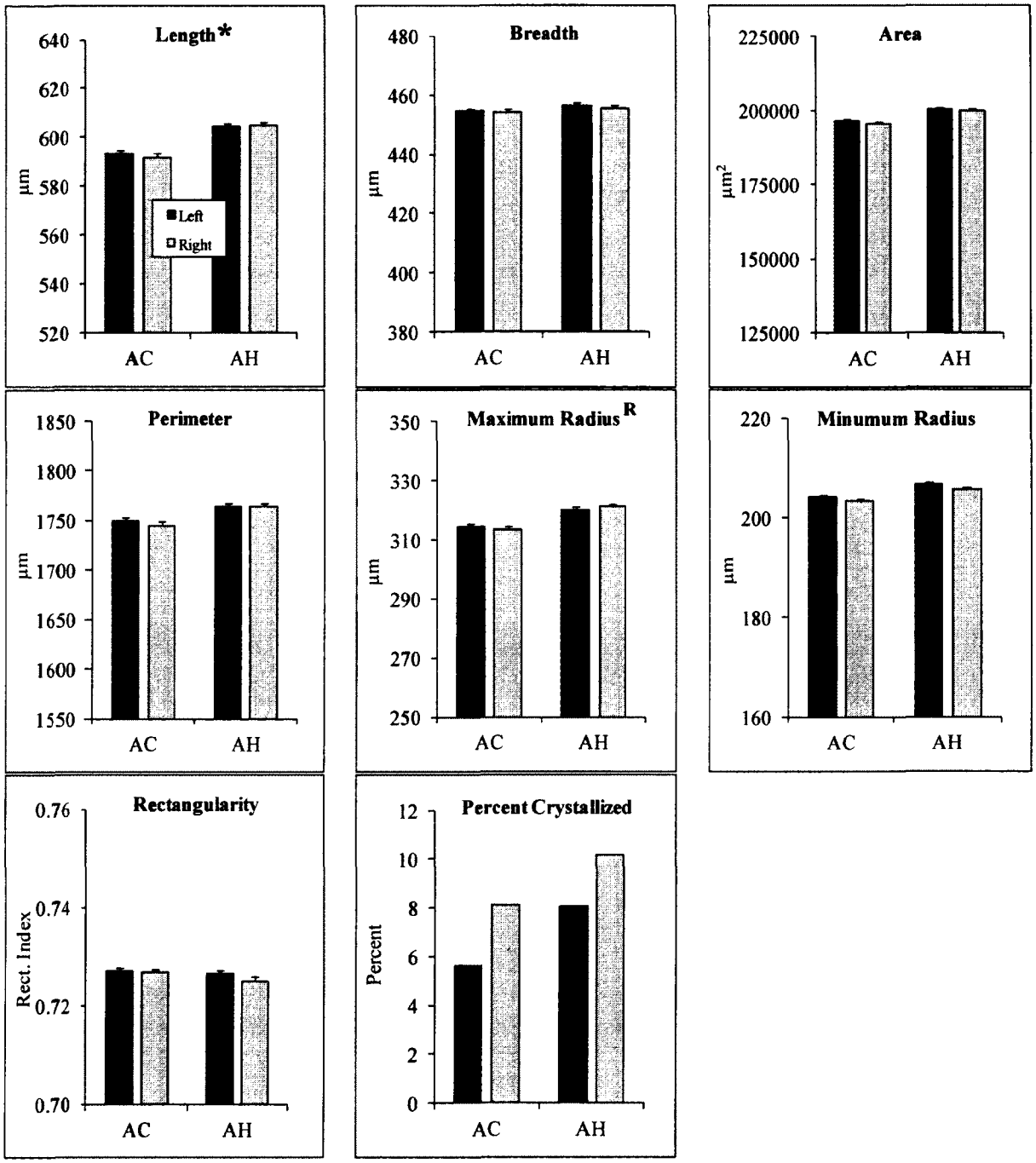

Figure 2.3. Measurements made on left (black) and right (grey) sagittal otoliths recovered from native pink salmon (AC) and their spatial hybrids (AH) reared in ambient temperature water, as well as the percentage of otoliths that were vateritic (crystallized). Error bars represent standard error. The (*) indicates significant differences between crosses for both left and right otoliths ($p \leq 0.01$). The (R) indicates the right otolith was affected.

CHAPTER 3

OTOLITHS AS AN INDICATOR OF DEVELOPMENTAL STABILITY IN PINK SALMON (*ONCORHYNCHUS GORBUSCHA*) EXPOSED TO ENVIRONMENTAL AND GENETIC STRESSORS[1]

[1] Oxman, D.S., Smoker, W.W., and Gharrett, A.J. In preparation. Otoliths as an indicator of developmental stability in pink salmon (*Oncorhynchus gorbuscha*) exposed to environmental and genetic stressors. Canadian Journal of Fisheries and Aquatic Sciences.

ABSTRACT

We examined the effects of possible environmental and genetic stressors (chilled water and outbreeding) on otolith formation in pink salmon. Full and half-sibling families of salmon from Auke Creek (Juneau, Alaska) and of outbred hybrids between Auke Creek females and Pillar Creek males from Kodiak Island, Alaska (1000 km distant) were incubated in ambient Auke Creek water. Native Auke Creek families were also incubated in chilled water. Trait-specific estimates of fluctuating asymmetry (FA) and cumulative indices of asymmetry (CFA) were compared among treatments to determine if potential stressors influenced otolith development. Trait-specific FA did not vary with environmental or genetic stressors, but CFAs indicated that cooler incubation temperatures were correlated with increased otolith asymmetry and phenotypic variability. Outbreeding had no affect on FA, but there was evidence of heterosis and increased phenotypic variability in some traits. Sire, dam, and interactions between parents did not affect otolith symmetry. Neither temperature nor outbreeding influenced the symmetry of otolith shape. Based on these results, otolith asymmetry can be used to detect environmentally-induced developmental stress in pink salmon, but it may not detect the effects of genetic stress with presently available tools.

Keywords: Developmental stability, otoliths, pink salmon

INTRODUCTION

Exposure to stressors can disrupt developmental pathways and produce phenotypic variability. In bilaterally symmetrical organisms, instability can be expressed as structural divergence between bilaterally paired structures and measured in terms of "fluctuating asymmetry" (FA), a variable that describes structural deviation from perfect symmetry (Palmer 1994). Biologists often use FA as an indicator of the effect of stress on developmental stability and population health (Palmer and Strobeck 2003).

In fishes, the relationship between FA and environmental stress is well studied. Several factors, which include pollution (Valentine et al. 1973), high or low temperatures (Soule 1982, Campbell 2003), climate change (Alados et al. 1993), and rearing location (Romanov and Parenskii 1997), produce developmental instability. Discovering relationships between genetic stressors, FA, and developmental instability, however, has been more difficult.

Although there is evidence that genetic stressors can influence symmetry in fish, the nature of the effect is often ambiguous (Green and Lochmann 2005). Several studies indicated that increased homozygosity, which resulted from inbreeding, correlated significantly with FA (Leary et al. 1985, Crozier 1997), but others have not (Panfili et al. 2005, Fessehaye et al. 2007). There is also ambiguity regarding the effects on symmetry of outbreeding (Wilkins et al. 1995, Gharrett et al. 1999). Failure to explain such contradictions may be due in part to our lack of understanding regarding the genetic transmission of symmetry and asymmetry. Clearly, the nature of the relationship between

genetics and developmental stability needs further investigation, especially among salmon populations for which enhancement programs increase the risk of inbreeding and outbreeding.

Several bilateral structures have been used to evaluate developmental stability in fish, including fin rays (Bryden and Heath 2000), gill rakers (Leary et al. 1985), and otoliths (Alados et al. 1993). Otoliths would seem to be an ideal trait because their development is influenced by both biological and physical factors (Campana 1999). Indeed, otolith FA has been correlated with stress (Alados et al. 1993, Anken et al. 1998), although the correlation is not universal; several studies failed to observe that stress influenced otolith symmetry (Panfili et al. 2005, Fey and Hare 2008). Consequently, the relationship between otolith FA and stress must be evaluated on a case by case basis before it is used as an indicator of instability.

The primary objective of our study was to determine if environmental or genetic stress influenced otolith symmetry in pink salmon (*Oncorhynchus gorbuscha*) and, if so, to evaluate the usefulness of otolith FA as an indicator of developmental stress. We also asked whether parental genomes individually or interactively influenced otolith symmetry. Several steps were taken to accomplish these objectives: (1) full- and half-sib families of Auke Creek (Juneau, Alaska) pink salmon embryos were incubated in ambient Auke Creek water to provide information about the parental effects on otolith formation; (2) some of these families were reared in chilled water for the first 14 weeks to determine how exposure to thermal stress early in development influenced otolith symmetry; and (3) third generation hybrids between spatially separated populations

(herein referred to as "spatial hybrids") were bred to see if outbreeding affected otolith development.

MATERIALS AND METHODS

Breeding and Incubation

Pink salmon were collected at Auke Creek Research Station (a facility of US National Oceanic and Atmospheric Administration's Alaska Fisheries Science Center) from a weir near the mouth of Auke Creek, a 350 m high-gradient, lake-fed stream located near Juneau, Alaska, a stream in which populations of pink salmon naturally spawn (Gilk et al., 2004). Mature salmon used in this experiment were fin-marked individuals that represented the second filial (F_2) generation of a breeding experiment involving native Auke Creek pink salmon and spatial hybrids that had been released as fry to the North Pacific Ocean from the Auke Creek Research Station during the spring of 1999. The hybrid lineage was originally created by crossing late-run females in Auke Creek with late-run males from Pillar Creek on Kodiak Island, Alaska (Gilk et al. 2004). Pillar Creek is a 1800 m long reservoir-fed stream located 1000 km west of Juneau that is historically 1 to 2 °C cooler than Auke Creek between late August when the salmon spawn and mid-November when their eggs hatch. Although the latitudes (near 58°N) and habitats of Auke and Pillar Creeks are similar, their spatial separation and different temperature regimes make it likely that the groups have evolved independently into

genetically distinct and locally adapted populations (Adkison 1995, Gilk et al. 2004). Consequently, crosses between these demes produced what we will refer to as spatial hybrids that allowed us to study the effect of outbreeding on otolith development.

In late August 2000, the F_2 generation of maturing salmon, descendants of the natives and hybrids bred in 1996 by Gilk et al (2004) and in 1998 by Wang et al (2007), were collected as they returned to Auke Creek, sorted according to gender and fin clip (native or hybrid), and held for several days until maturation was complete. On 3 September 2000, a blocked 2x2 factorial design was used to produce full- and half-sib F_3 families from native (20 males and 20 females) and hybrid (16 males and 16 females) returning fish. There were 10 blocks of natives (40 families) and 8 blocks of hybrids (32 families).

Fertilized eggs were incubated in divided trays (FAL^{TM}; Marisource Milton, WA) that housed each family separately. Each tray had 10 compartments which contained single families. These trays were placed in incubation cabinets so that each cabinet contained eggs from a single type of cross (native or hybrid). The eggs in one native and one hybrid cabinet were incubated in ambient temperature Auke Creek water. Because water temperatures in the stream, intragravel environment (redds), and research station are similar, development of natives reared under ambient conditions represented the normal development of wild Auke Creek pink salmon. These natives were the control group. A subset of eggs from each of the native families was placed in a third cabinet and incubated in a simulated environment that was, on average, 2.4 °C (± 0.9 °C) cooler per day than ambient Auke Creek stream temperatures through hatching (Fig. 3.1). All

families were divided into four approximately equal portions, two of which were randomly assigned to compartments within a cabinet in each temperature regime to provide replication.

The temperature in the simulated environment was altered daily to mimic natural variation in incubation conditions (Fig. 3.1). Water in the cold regime was re-circulated to achieve the level of control needed to simulate daily thermal fluctuations in a captive setting. Two 1,136-liter Living Streams® (Frigid Units, Inc. Toledo, OH) were used as a reservoir and equipped with a 1-hp chiller. A ¾-hp submersible pump was used to move water from the reservoir to a 76-liter head tank, which distributed water to the incubation cabinets by a gravity feed. Water from the incubators drained into the reservoir, where it was conditioned and pumped back to the head tank. Water in the reservoirs was continually replaced with fresh stream water; a complete turnover occurred every 5 hours. The experiment was designed to determine if exposure to thermal stress early in development affected otolith symmetry. Consequently, the water was not chilled after the 14^{th} week of development when ambient temperature Auke Creek water was allowed to flow unidirectionally and water temperatures in the two regimes were identical (Fig. 3.1).

Water was supplied to all incubators at a rate of 8 L/min until the eyed stage was reached, and at 23 L/min thereafter. Temperatures were recorded once per day to the nearest 0.1 °C and did not differ substantially between incubation cabinets for a particular temperature regime. Before hatching, incubating eggs were treated once a week with formalin (1:6000 in static water) for one hour to reduce infestations by fungus and algae, a standard practice in the culture of salmon embryos. After hatching, weekly salt

treatments (3 parts per thousand sodium chloride for one hour) were used to minimize infestations, another common practice in salmon culture. All water was filtered through sheer nylon mesh prior to entry into the incubation cabinets to prevent hydra infestation.

Otolith Measurements and FA Calculations

Fry were collected after yolk absorption from all families. By sampling fry after yolk consumption was complete, we ensured that development was standardized across treatment groups and controlled for age-related bias. Six siblings per replicate (12 fry per family) were sampled from each cross and temperature regime. The standard length (SL) for each of individual was recorded. This sampling scheme produced three treatment groups: ambient natives (i.e. controls: (AC), cold natives (CC) and ambient hybrids (AH)).

Sagittal otoliths were removed from each fry, mounted sulcus-side up on glass slides in thermoplastic cement, and photographed with a hi-resolution 5 megapixel (2592 x 1944 pixels) Leica DFC420 digital camera. The system was calibrated at 25X magnification with a slide micrometer. Measurements were recorded to the nearest micron. Six characteristics were quantified for each left and right otolith by using Optimas 6.5 Image Analysis software: area, perimeter, longest length, breadth, maximum radius, and minimum radius. Radii measurements originated from the otolith's center of mass, which was located in the central-most region of the otolith where multiple primordia fused to form the otolith's core. Since the longest length transect seldom

passed through the center of mass, radii measurements did not coincide with the longest length transect. A rectangularity index, defined as the otolith area divided by the area of an enclosing bounding box oriented along the otolith's major axis (Optimas 1996), was used to describe otolith shape. An index of 0.5 indicated that the boundary box was square. The index increases as the boundary box becomes more rectangular and an index of 1.0 is a line.

These seven attributes were chosen to quantify otolith asymmetry because they were likely to exhibit some degree of covariance, and co-dependent variables provide a more robust estimate of individual asymmetry (Palmer 1994). Although all aspects of otolith morphology are structurally linked, their development may not be entirely co-dependent because growth rates vary considerably over the surface of the otolith (Mugiya 1968, Ibsch et al. 2004). Pearson correlation coefficients were used to determine which of the measured traits were correlated.

Fluctuating asymmetry, defined as the absolute value of the difference between the right and left side of a bilateral trait, was used to quantify the developmental stability of each otolith attribute. We combined the FAs of multiple traits into a single composite measure of asymmetry (CFA) for each individual. This composite index can be more sensitive than a single-trait quantification of FA because the individual FAs may exhibit low levels of variation and thereby provide a weak estimate of the underlying developmental instability (Palmer 1994, Leung et al. 2000). The CFA was defined as:

$$(1) \quad CFA1_i = \sum_j \left[\frac{|R_{ij} - L_{ij}|}{|\overline{R_j - L_j}|} \right]$$

where R_{ij} is the measurement of trait j from fish i's right side, L_{ij} is the corresponding measurement from the left side, and $R_j - L_j$ is the average FA for trait j. (Leung et al. 2000). By expressing the asymmetry of each trait within an individual relative to the average asymmetry of that trait for the entire sample, the index removed size-dependent differences in FA among traits. An alternative equation that removed size-dependent differences at the individual level was also estimated (Palmer and Strobeck 2003):

$$(2) \quad CFA2_i = \sum_j \left[\frac{| R_{ij} - L_{ij} |}{(R_{ij} + L_{ij})/2} \right]$$

Statistical Analysis

Traits with non-normal FA distributions may not provide reliable measures of developmental stability because they do not conform to the pattern of bilateral variation that is expected to result from developmental instability (Palmer and Strobeck 2003). Developmental instability results from the accumulation of subtle, random variation in developmental processes that influence each side independently, so it should generate normal distributions of FA. Although developmental instability may occasionally generate non-normal FA distributions, the presence of such distributions may indicate that other processes are influencing development (Palmer and Strobeck 1997). Antisymmetry and directional asymmetry (DA), for example, could be caused by established genetic pathways as well as by developmental perturbations (Palmer 1994). As a result, traits whose FA deviate from normality may not provide reliable measures of

developmental stability because the cause of their asymmetry is uncertain. Departures from normality can also generate bias by inflating estimates of FA (Palmer 1994). Consequently, attributes with non-normal FA distributions were identified and excluded from the evaluation of developmental stability to avoid potential bias (Palmer and Strobeck 1997). Student's *t*-tests of skewness and kurtosis for normality, as well as one-sample Student's *t*-tests for DA, were conducted prior to the analysis of FA. Traits that exhibited significant antisymmetry or DA in any single treatment group were excluded from all FA and CFA calculations so that the same traits were used to calculate CFAs for all three treatment groups.

Restricted maximum likelihood analysis (REML) was used to determine how parents, thermal stress, and outbreeding influenced the developmental stability of sagittal otoliths. REML is more robust to deviations from normality, non-homogenous variances, and unbalanced designs in mixed-model comparisons than the ANOVA (Lynch and Walsh 1998, Van Dongen et al. 1999). The REML method uses the Z-statistic, defined as the estimated covariance parameter divided by its approximate standard error, to test random effects for significance. The *F*-statistic is used to evaluate fixed effects. Asymmetry was compared among families within a treatment with the following REML model:

$$(3) \quad Y_{klmno} = \mu + B_k + D_{kl} + S_{km} + D_{kl}*S_{km} + R_{klmn} + \varepsilon_{klmno}$$

where Y_{klmno} was the dependent variable (FA or CFA), μ was the population mean, B_k was the effect of block (i.e. the independence and randomness of the experimental design), D_{kl} was the effect of dam, S_{km} was the effect of sire, $D_{kl}*S_{km}$ was the effect of the interaction

between dam and sire (i.e. family effects), R_{klmn} was the effect of compartment position on development (i.e. microhabitat effects), and ε_{klmno} was the residual random error (i.e. variation within families). All factors in this equation were random.

The following REML model was used to test the effects of temperature on otolith morphology:

$$(4) \quad Y_{jklmno} = \mu + T_j + B_k + T_j*B_k + D_{kl} + S_{km} + D_{kl}*S_{km} + T_j*D_{kl} + T_j*S_{km} + T_j*D_{kl}*S_{km} + R_{jklmn} + \varepsilon_{jklmno}$$

where T_j was the effect of incubation temperature, T_j*B_k was the effect of the interaction between temperature and block (i.e. the effect of temperature on the randomness and independence of the experimental design), T_j*D_{kl} was the effect of the interaction between temperature and dam (i.e. environmental interactions related to dam effects), T_j*S_{km} was the effect of the interaction between temperature and sire (i.e. genotype-by-environment (GxE) interactions related to sire effects), and $T_j*D_{kl}*S_{km}$ was the interaction between temperature and the family effect (i.e. GxE interactions related to the family effect). The equation used to determine if outbreeding affected developmental stability was defined as:

$$(5) \quad CFA_{iklmno} = \mu + C_i + B_{ik} + D_{ikl} + S_{ikm} + D_{ikl}*S_{ikm} + R_{iklmn} + \varepsilon_{iklmno}$$

where C_i was the effect of cross. Equations 4 and 5 were mixed-models because they included both random (block, dam, sire, replicates) and fixed (cross, temperature) effects.

Another potential result of developmental instability that may arise from either genetic or thermal stress is increased phenotypic variation (Palmer and Strobeck 2003). Therefore, the variation in normally distributed trait-specific FA was also compared

among treatments with Levene's test for homogeneous variances to determine if treatment increased phenotypic variability. Increased variation in a trait's asymmetry was considered an indicator of disrupted development.

Because the relationships within and among families were known, we used a quantitative genetics approach to express the variability partitioned by each REML model in terms of its underlying causal genetic factors (Lynch and Walsh 1998). Given our breeding design, the variation in FA associated with sire effects would imply that the heritable source of such variability came mostly from additive genetic factors (Table 3.1). Dam-associated variability also would primarily provide evidence of an additive effect and maternally inherited effects such as common maternal environmental influences which include egg quality and egg size (Table 3.1). Interactions between dam and sire would imply that variation in FA came from non-additive genetic sources such as dominance and/or epistatic effects (Table 3.1). Therefore, by using REML to test for significant dam, sire, and family effects, we evaluated the underlying genetic components of variation in developmental stability.

All comparisons and hypothesis tests were conducted in SAS 9.1with PROC MIXED procedures (SAS Institute, 2002). Both single-trait FA measurements and CFA indices were log-transformed to normalize residuals. The significance level used for hypothesis testing was $p \leq 0.05$. Native fry reared at ambient temperature (controls) were used to establish the normal range of asymmetry and variability of the reference population (i.e. Auke Creek pink salmon). Otolith asymmetry among native fry incubated in cold water, which exceeded that of natives raised at ambient temperature, was

presumed to indicate that developmental processes were thermally disrupted. Similarly, asymmetry observed in spatial hybrids which exceeded that of controls was presumed to reflect the genetic differences between hybrids and natives and indicative of outbreeding.

RESULTS

In total, 475 pairs of otoliths were recovered from both AC and CC fry, and 379 pairs were collected from AH fry. Area, perimeter, and minimum radius measurements were either non-normal or had significant directional asymmetry that emphasized the left side and were excluded from our FA and CFA calculations for all three treatment groups (Table 3.2). The left-sided DA was present in native fish reared at ambient temperatures, but it was virtually absent from their thermally stressed counterparts and outbred hybrids (Table 3.2). Specifically, DA values in AC fry differed significantly from zero ($p \leq 0.049$) because the area, perimeter, and minimum radius of left otoliths were larger than right otoliths.

Otolith length, breadth, maximum radius, and rectangularity were normally distributed ($p \geq 0.090$) and lacked directional asymmetry in all treatments ($p \geq 0.080$). Consequently, these traits were used to calculate FA and CFA indices. These attributes were correlated with each other in both left and right sides in all treatments ($p \leq 0.010$), with the exception of rectangularity and breadth on the left otolith of hybrid fry ($p \geq 0.095$). The strength of these relationships varied among pairwise comparisons, with correlation coefficients ranging between -0.280 and 0.968 (Table 3.3). The correlations

between rectangularity and the other otolith traits were weak (Table 3.3). As a result, it was excluded from the CFA calculation because the sensitivity of the index depends on the strength of these correlations (Palmer and Strobeck 2003).

Because correlations between the otolith measurements and fish length were weak (Table 3.3) and fry length (SL) did not differ substantially among treatments (≤ 0.4 mm), trait-specific FA values were not standardized by SL. Similarities in SL among treatments also meant that any differences in otolith structure among groups could not be attributed to differences in fish length.

Native Auke Creek Fry (Controls)

All aspects of otolith morphology exhibited some degree of asymmetry in native fry reared at ambient temperatures (Fig. 3.2). The largest asymmetry was associated with otolith length (FA = 16 ± 12 μm), whereas the maximum radius measurement had the least FA (11 ± 8 μm; Fig. 3.2). Length, radius, and breadth FA values were combined to produce a CFA1 index of 2.81 ± 1.68 and a CFA2 index of 0.09 ± 0.05 (Fig. 3.3). Otoliths were typically rectangular (rectangularity index = 0.73 ± 0.02) and this shape was expressed in left and right otoliths with relative consistency (FA = 0.02 ± 0.01).

Comparisons of single trait FA and CFA1 among AC families indicated that neither dam effects, sire effects, nor family effects influenced otolith formation in terms of its symmetry (Table 3.4). Because otolith development can be responsive to small-scale environmental changes (Volk et al. 1999, Barnett-Johnson et al. 2008), we also

compared FA and CFA1 among compartments to determine if a family's position within the incubator influenced otolith symmetry. We observed, however, that microhabitat had no measurable effect on otolith development (Table 3.4). Similar results were observed with the CFA2 index.

Temperature Effects

Otoliths from fry reared in cold water were compared to those of fry raised at ambient temperatures to determine if thermal stress affected their developmental stability. The FA associated with the length, breadth, and maximum radius of otoliths from CC fry increased by 18.5%, 12.4%, and 12.4%, respectively, relative to that of AC fry (Fig. 3.2). Except for breadth ($p = 0.021$), these differences were not significant (Table. 3.4). Levene's test indicated variability associated with these FA values, however, was higher among the fry reared in cooler waters ($p \leq 0.001$; Fig. 3.2).

Trait-specific increases in FA among CC fry resulted in significant increases in both CFA1 (14.4%; $p = 0.049$) and CFA2 (18.7%; $p = 0.018$: Fig. 3.3). Incubation temperature, in contrast, did not alter the FA associated with otolith shape as defined by the rectangularity index ($p = 0.107$), although it did result in increased variability (Levene's test; $p \leq 0.001$; Fig. 3.2).

Comparisons of FA and CFA indices among families and treatments indicated that no genotype-by-environment effects were associated with dam, sire, or family effects

(Table 3.4). In addition, the position of a family of native fry within the cold incubation stacks did not influence any aspect of otolith development (Table 3.4).

Hybridization Effects

Otoliths from native pink salmon and their spatial hybrids incubated at ambient temperatures were compared to determine if outbreeding influenced the developmental stability of otoliths. Otolith length and maximum radius FA were 14.4% and 17.7% larger, respectively, in hybrids than in their native counterparts (Fig. 3.2). Although these differences were not significant (Table 3.4), the Levene's Test indicated variability of these trait-specific FA estimates was higher among AH fry ($p \leq 0.001$). Breadth, in contrast, was more symmetrical in hybrids; its FA was 13.0% lower relative to controls (Fig. 3.2). This increased symmetry was not significant ($p = 0.110$) and there was no change in its variability (Levene's Test; $p = 0.070$; Fig. 3.2).

Because composite asymmetry calculations assume all traits are affected by stress in the same way, breadth was omitted from both CFA indices. The CFA1 index for hybrids was 16.1% larger than that of controls (Fig. 3.4). This difference, however, was not significant ($p = 0.134$). The CFA2 index was 13.6% larger in AH fry, but this increase was also insignificant ($p = 0.236$; Fig. 3.4).

Hybridization did not influence otolith shape FA ($p = 0.174$), nor did it increase its variability (Levene's test; $p = 0.086$). Like breadth, shape symmetry was more consistent among hybrid fry: rectangularity FA estimates were 11.1% smaller than those

of native fry (Fig. 3.2). The position of a hybrid family within the incubation stack did not influence otolith development (Table 3.4).

DISCUSSION

Although there were several indications that otolith development was sensitive to stress, the sensitivity appeared to depend on the type of stressor. Comparisons between incubation environments indicated that thermal stress correlated with otolith asymmetry and increased phenotypic variation, both of which are indicative of developmental instability (Valentine et al. 1973, Palmer and Strobeck 2003). Thermal disruption of developmental pathways was not unexpected since fish are ectothermic and as such, their metabolic processes are regulated to some degree by the external environment. Indeed, changes in water chemistry and temperature have been correlated with changes in otolith morphology in numerous species (Alados et al. 1993, Volk et al. 1999, Barnett-Johnson et al. 2008). It is this sensitivity to external variables that led many to believe that otoliths would be suitable for detecting environmentally-induced developmental instability (Radhakrishnan et al. 2009). Our results support this perspective because all aspects of otolith morphology, with the exception of shape FA, were influenced by thermal stress.

Genetic stress would be expected to generate developmental instability because genes play a significant role in the regulation of fish metabolism and development. Our results, however, were ambiguous. The increased phenotypic variation associated with otolith length and radius suggested that spatial hybridization had disrupted developmental

pathways, but the CFAs indicated that net otolith formation was unaffected by the genetic disruption. This relative stability could have been the result of genetic similarities between stocks, genetic and developmental flexibility (plasticity) in the source populations, or canalization, though none of these possibilities are mutually exclusive.

Canalization tends to be associated with fitness-related traits (Stearns et al. 1995, Gaillard and Yoccoz 2003); and because shape and symmetry are important to otolith function, which is essential to fish survival (Tomas and Geffen 2003, Panfili et al. 2005, Gagliano et al. 2008), it is not surprising that the otolith phenotype would be developmentally stable. The symmetry of shape seemed to be particularly well canalized since it was unaffected by either incubation temperature or spatial hybridization. This genetic conservation of shape symmetry is reinforced by the fact that neither dam, sire, nor interaction between dam and sire had significant effects on otolith growth. Genotype-by-environment effects were also lacking, suggesting locally adapted genes did not substantially influence otolith development and that the genes that govern otolith formation may be similar among stocks.

Adding to the uncertainty associated with genetic effects was the observation that spatial hybridization increased the symmetry of two otolith attributes: breadth and rectangularity. Reduced asymmetry may indicate heterosis, which can produce phenotypic stability as a consequence of the increased genetic variability that results from hybridization. When viewed in this context, the presence of heterosis indicates genetic modification.

Although traits with directional asymmetry were omitted from the FA and CFA analyses because they differ in nature from FA, which is usually used to evaluate developmental stability (Palmer and Strobeck 1986), their occurrence did provide some insight. Inner ear function depends on otolith symmetry (Gagliano et al. 2008); however, otolith area, perimeter, and minimum radius were consistently and significantly larger in left otoliths of native fish reared in ambient temperatures. Aspects of otolith morphology that had insignificant DA also tended to exhibit left-sided bias. From this perspective, the presence of DA in the control group seems to indicate that development was disrupted, possibly as a result of being reared in a captive environment. If, on the other hand, it is assumed that the DA in controls is representative of normal development, then its virtual absence from hybrids and thermally-stressed larvae could also be considered indicative of altered development. In either case, it is clear that the presence of DA should be considered when evaluating developmental stability.

The relationships among traits and the variability of their reaction to stress made our analytical approach conservative. High levels of variation in trait-specific FA made it difficult to determine if temperature and hybridization influenced the stability of otolith development. Combining them into cumulative indices of asymmetry created parameters that were sufficiently sensitive to detect differences in developmental stability, but the correlations among otolith measurements were mostly weak, which resulted in conservative estimates of cumulative asymmetry (Palmer 1994). The fact that only two or three traits were available to formulate the CFA likely added to this conservatism since several traits were omitted from the analysis because they exhibited directional

asymmetry, antisymmetry, or violated the assumption that stress affected the development of each trait in the same way. Despite these limitations, the CFA indices yielded similar results and were still sufficiently sensitive enough to detect changes in overall otolith symmetry, thereby indicating that trait-specific FA and CFA provided robust evaluations of developmental stability.

While it is clear that otolith asymmetry may be useful for the detection of environmental stress in pink salmon, its usefulness for detecting the effects of genetic perturbations is uncertain and warrants further evaluation. The affects of other sources of genetic stress should be examined during earlier generations to determine if it influences otolith development, and additional otolith traits need to be evaluated to increase the robustness of the CFA index. Increasing the number of traits from which the CFA is estimated might improve its ability to detect developmental instability. It is important to note that the relationship between otolith asymmetry and stress was strictly correlative, not causative, and that instability associated with otolith formation may not be indicative of systemic instability. Ideally, the FA of unrelated bilateral traits should also be considered when evaluating an organism's overall developmental stability.

When our results are considered within the context of studies of otoliths from other species, it is evident that otoliths do not respond to stress the same way in all fishes. In some species, otolith asymmetry is correlated with stress exposure (Alados et al. 1993, Green and Lochmann 2005), whereas in others it is not (Panfili et al. 2005). Similarly, various aspects of otolith morphology can react differently to stress within a species (Green and Lochmann 2005, this study). This lack of consistency within and among

species suggests an otolith's developmental response to stress may be trait and/or species-specific. Consequently, an otolith's developmental response to stress should be thoroughly evaluated on a case by case basis before it can be used to evaluate developmental stability.

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Table 3.1. Genetic components estimated from the phenotypic variation observed among the offspring produced from a 2x2 factorial breeding design; V_A is the additive genetic variance (e.g. the variation caused by the influence of numerous alleles that combine to have a cumulative effect on phenotypic expression), V_D is the dominance genetic variance (e.g. the variation in expression caused by dominance effects), V_{AA} is the variance associated with additive x additive epistasis, V_{AD} is the variability deriving from additive x dominance epistasis, V_{DD} is the variability from dominance x dominance epistasis, and V_M is the maternal effects variance that derived from maternal effects like egg quality and size. High order effects include sources of genetic variation deriving from epistatic interactions that involve three or more loci. The asterisk denotes interaction.

Observed Phenotypic Variance	Genetic and Environmental Interpretation
Sire (σ_S^2)	$^1/_4V_A + {}^1/_{16}V_{AA}$ + Higher Order Effects
Dam (σ_D^2)	$^1/_4V_A + {}^1/_{16}V_{AA} + V_M$ + Higher Order Effects
Dam * Sire (σ_{DS}^2)	$^1/_4V_D + {}^1/_8V_{AA} + {}^1/_8V_{AD} + {}^1/_{16}V_{DD}$ + Higher Order Effects

Table 3.2. Otolith measurements that exhibited significant directional asymmetry (DA) and/or deviations from normality. The presence of DA was tested for each trait with a one-sample *t*-test to determine if the mean (R – L) differed significantly from zero. Departure from normality (i.e. antisymmetry) was evaluated with *t*-tests of skewness and kurtosis. The "--" indicate no significant DA or antisymmetry was observed. All other categories were significant ($p \leq 0.049$). These measurements were not used to evaluate fluctuating asymmetry in any of the treatment groups.

	Area	**Perimeter**	**Min. Radius**
Directional Asymmetry			
Ambient Natives (Controls)	Left-sided	Left-sided	Left-sided
Cold Natives	--	--	--
Ambient Hybrids	--	--	Left-sided
Skewness			
Ambient Natives	--	Skewed Left	Skewed Left
Cold Natives	--	--	--
Ambient Hybrids	--	--	Skewed Left
Kurtosis			
Ambient Natives	--	Leptokurtic	Leptokurtic
Cold Natives	Leptokurtic	Leptokurtic	Leptokurtic
Ambient Hybrids	Leptokurtic	--	Leptokurtic

Table 3.3. Pearson correlation coefficients for measurements of sagittal otoliths from native (AC) and hybrid (AH) fry reared in ambient water, as well as from natives incubated in cold water (CC) at Auke Creek Hatchery. An asterisk indicates the correlation was not significant ($p \geq 0.052$). SL = standard length.

	Length	Breadth	Rectangularity	Max. Radius
AC: Right Otolith				
Breadth	0.560	--	--	--
Rectangularity	-0.213	-0.336	--	--
Max. Radius	0.967	0.488	-0.262	--
SL	0.353	0.494	-0.026*	0.311
AC: Left Otolith				
Breadth	0.546	--	--	--
Rectangularity	-0.188	-0.291	--	--
Max. Radius	0.968	0.473	-0.236	--
SL	0.406	0.551	-0.131	0.357
CC: Right Otolith				
Breadth	0.500	--	--	--
Rectangularity	-0.244	-0.461	--	--
Max. Radius	0.960	0.464	-0.267	--
SL	0.340	0.447	-0.092*	0.314
CC: Left Otolith				
Breadth	0.456	--	--	--
Rectangularity	-0.219	-0.487	--	--
Max. Radius	0.953	0.436	-0.260	--
SL	0.394	0.474	-0.118	0.382
AH: Right Otolith				
Breadth	0.299	--	--	--
Rectangularity	-0.156	-0.151	--	--
Max. Radius	0.959	0.196	-0.280	--
SL	0.280	0.460	0.104*	0.197
AH: Left Otolith				
Breadth	0.260	--	--	--
Rectangularity	0.161	-0.086*	--	--
Max. Radius	0.960	0.155	-0.249	--
SL	0.251	0.440	0.150	0.189

Table 3.4. Probability values from REML models testing for the effects of dam, sire, family, incubation temperature, and hybridization on the fluctuating asymmetry of otoliths from native Auke Creek pink salmon and their spatial hybrids. The compartment effect evaluated the influence of a family's location within an incubation tray on otolith symmetry. "Rect." refers to the rectangularity shape index and "CFA1" and "CFA2" refer to the composite indices of fluctuating asymmetry. Grey boxes indicate significant effects ($p \leq 0.05$).

	Length	Breadth	Max. Radius	Rect.	CFA1	CFA2
Native Auke Creek						
Dam	0.724	0.993	0.255	0.990	0.986	0.932
Sire	0.998	0.501	0.497	0.915	0.448	0.411
Dam*Sire	0.931	0.664	0.804	0.852	0.520	0.486
Compartment	0.525	0.826	0.170	0.169	0.295	0.287
Temperature Effects						
Temp	0.116	0.021	0.252	0.107	0.049	0.018
Dam	0.429	0.341	0.934	0.835	0.554	0.883
Sire	0.962	0.579	0.744	0.851	0.884	0.867
Dam*Sire	0.801	0.714	0.606	0.995	0.932	0.939
Temp*Dam	0.792	0.209	0.606	0.826	0.468	0.627
Temp*Sire	0.664	0.877	0.899	0.394	0.448	0.428
Temp*Dam*Sire	0.949	0.676	0.431	0.750	0.909	0.993
Compartment	0.714	0.269	0.896	0.420	0.664	0.691
Hybridization Effects						
Cross	0.138	0.110	0.371	0.174	0.134	0.236
Dam	0.356	0.168	0.208	0.264	0.453	0.508
Sire	0.438	0.101	0.393	0.711	0.654	0.657
Dam*Sire	0.396	0.211	0.243	0.943	0.693	0.711
Compartment	0.839	0.053	0.786	0.367	0.247	0.269

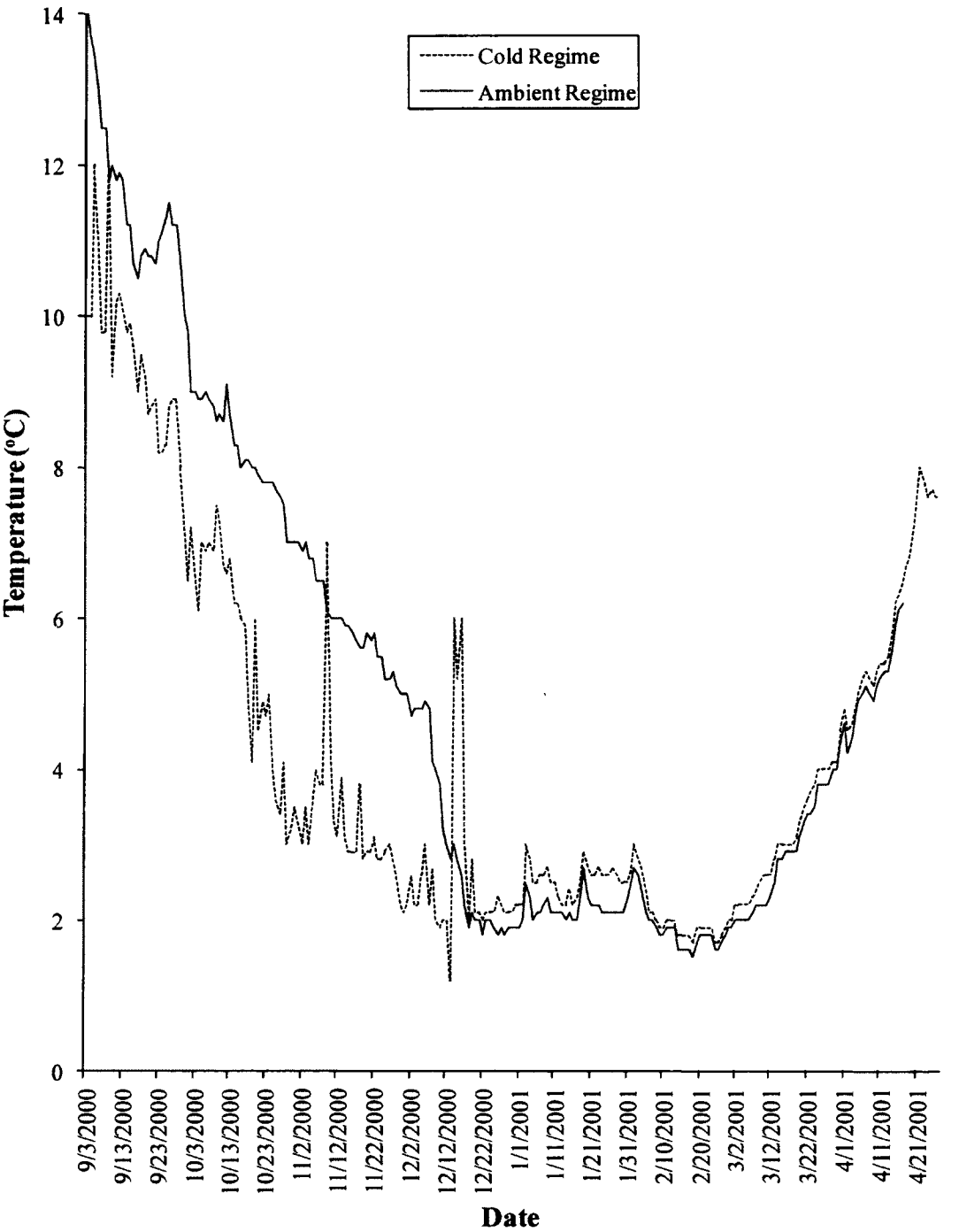

Figure 3.1. Water temperatures for two rearing environments for brood year 2000 pink salmon reared at Auke Creek Hatchery near Juneau, Alaska from 9/3/00 through 4/27/01. Ambient temperatures represent Auke Creek stream temperatures, whereas the cold regime was designed to mimic the natural variation of a cooler incubation environment. Water chillers were taken offline on 12/13/00.

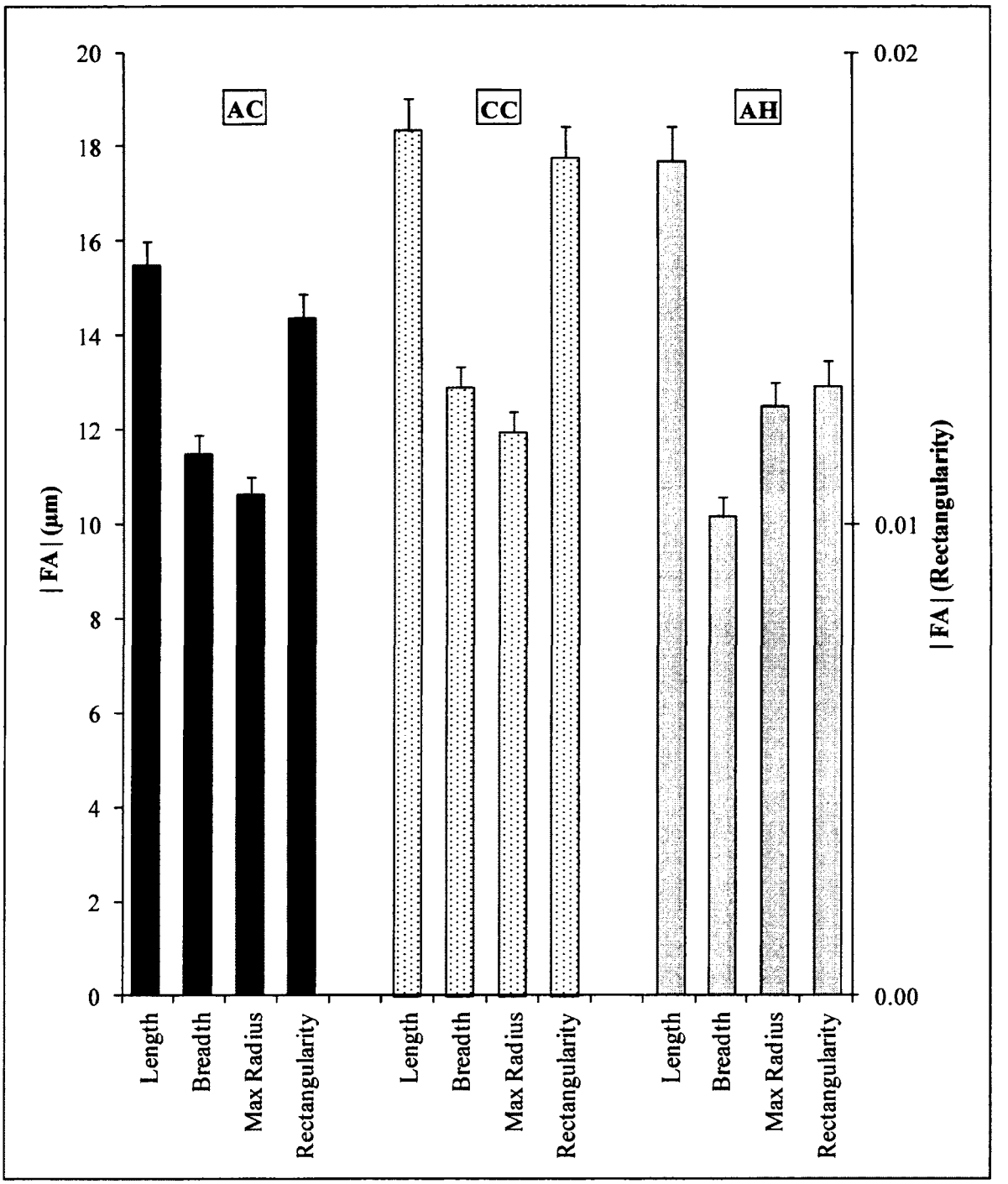

Figure 3.2. Absolute asymmetry values (|FA| = Left Side - Right Side) for each measurement made on sagittal otoliths recovered from native pink salmon reared in ambient (AC) and cold waters (CC), as well as hybrids incubated at ambient temperatures (AH). The secondary y-axis relates to the FA values associated with the rectangularity index. Error bars represent standard errors.

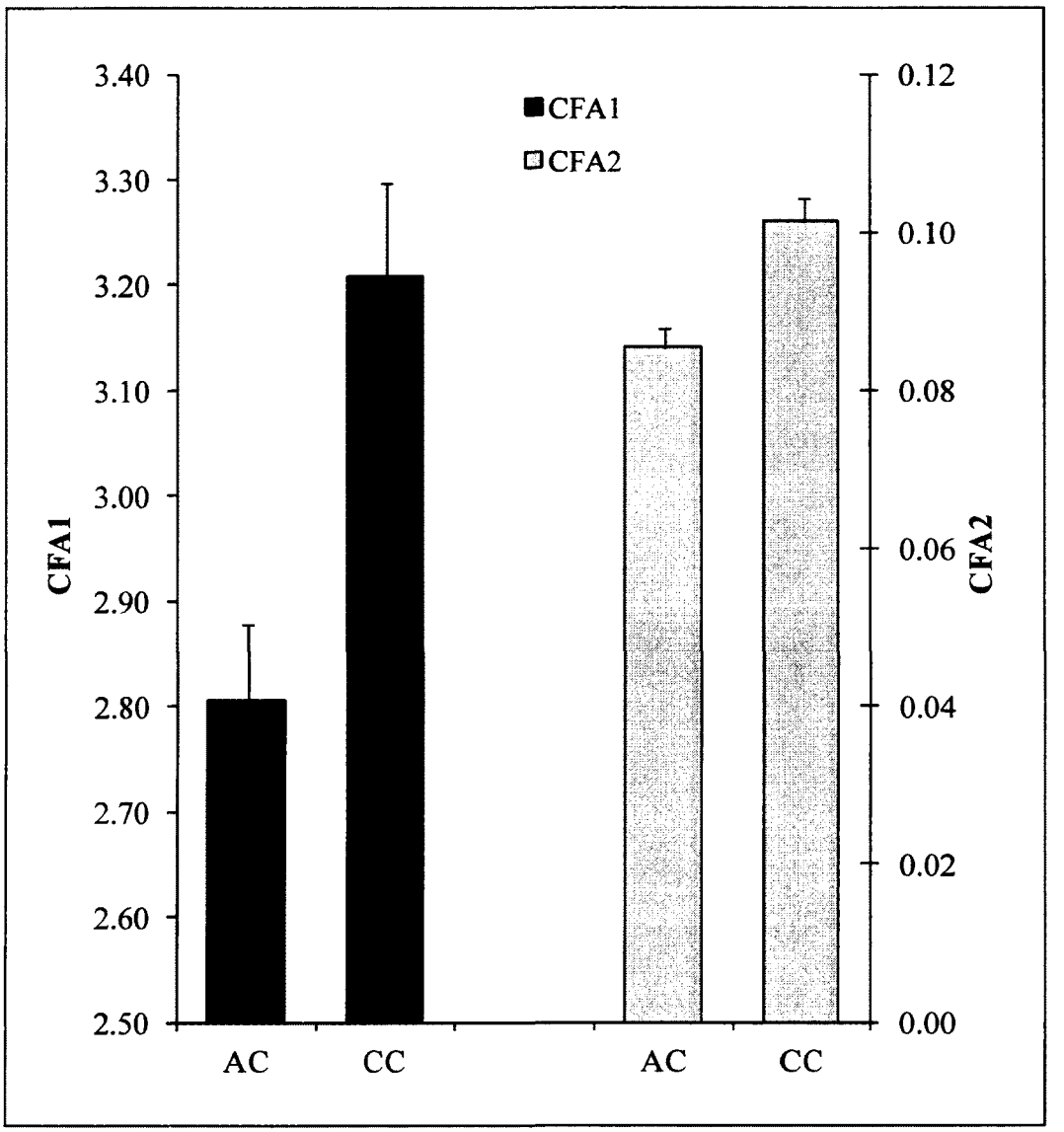

Figure 3.3. Two composite indices of fluctuating asymmetry (CFA) for native Auke Creek pink salmon reared in ambient temperature Auke Creek water (AC) and colder water (CC). Equations 1 and 2 (see text) were used to calculate CFA1 and CFA2, respectively. Both indices included otolith length, breadth, and maximum radius measurements. Error bars represent standard errors.

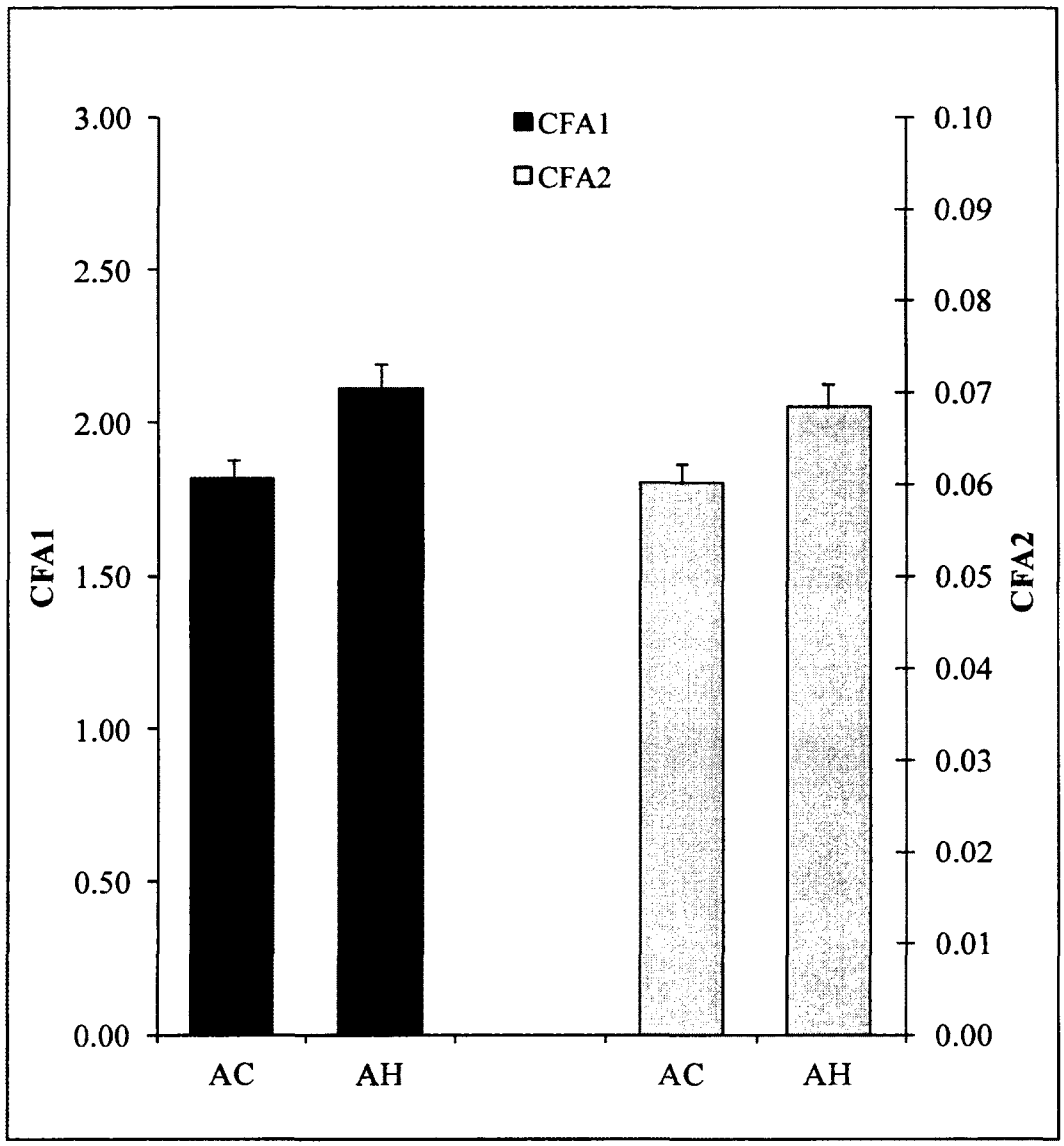

Figure 3.4. Two composite indices of fluctuating asymmetry (CFA) for native Auke Creek pink salmon (AC) and their spatial hybrids (AH) that were incubated in ambient temperature Auke Creek water. Equations 1 and 2 (see text) were used to calculate CFA1 and CFA2, respectively. Both indices included otolith length and maximum radius measurements. Error bars represent standard errors.

CHAPTER 4

DEVELOPMENTAL PROGRESSION OF GILL RAKERS AS A POST-HATCH DEVELOPMENTAL MARKER IN PINK SALMON (*ONCORHYNCHUS GORBUSCHA*)[1]

[1] Oxman, D.S., Smoker, W.W., and Gharrett, A.J. In review. Developmental progression of gill rakers as a post-hatch developmental marker in pink salmon (*Oncorhynchus gorbuscha*). Environmental Biology of Fishes.

ABSTRACT

We evaluate the usefulness of gill rakers as a post-hatch developmental marker in salmon by tracking development in undisturbed and stressed yolk-bearing salmon embryos. Native pink salmon (*Oncorhynchus gorbuscha*) from Auke Creek, Juneau, Alaska and outbred hybrids between Auke Creek and Pillar Creek (Kodiak Island) salmon were incubated in ambient-temperature Auke Creek water. Environmentally stressed native embryos were reared in water that was 2 to 4 °C warmer than ambient. The sum of rakers on the first left and right branchial arches of natives reared at ambient temperatures averaged 23.20 (SD ± 1.64) per embryo when post-hatch sampling began. The subsequent increase in raker counts was linear and positively correlated with the accumulation of thermal units until counts reached maxima 223 days after fertilization, which coincided with the complete consumption of yolk reserves. The average maximum raker count was 39.55 (SD ± 1.76) per embryo, which is substantially fewer than the 60 rakers typically observed in adults. Apparently, raker growth ceased because yolk reserves were depleted and not because raker formation was complete. Neither raker development nor yolk consumption patterns were affected by hybridization. Elevated incubation temperatures accelerated raker formation and yolk consumption in native embryos, but the number of rakers associated with a given amount of yolk was the same regardless of temperature suggesting that changes in yolk consumption rate, and raker development rate did not influence raker counts. These results indicate that rakers are easily observed and counted, grow in a predictable sequence, and are developmentally

stable in the face of both genetic and environmental stress, thereby making them potentially reliable post-hatch developmental markers.

Key Words: Developmental sequence, salmonids, gill rakers; ontogeny

INTRODUCTION

The progressive development of morphological traits is often used to identify, describe, and characterize developmental states, especially in species like Pacific salmon (*Oncorhynchus* spp.), which pass through numerous stages during the course of their life history (Groot and Margolis 1991). The identification of developmental landmarks is important for identifying species-specific developmental features (Haddon and Willis 1995; Foote et al. 1999), differentiating among species during larval stages (Bornbusch and Lee 1992; Neira et al. 1998), evaluating phylogenetic relationships (Bornbusch and Lee 1992; Britz 1997), and evaluating aquacultural protocols (Pelluet 1944; Koumoundouros et al. 1994).

The developmental milestones that define normal stages of pre-hatch development in salmonids have been well documented (Battle 1944; Ballard 1973). Embryonic stages have been described by Ballard's scale (Ballard 1973), which consists of 30 stages divided among three developmental phases: cleavage (cell division), epiboly (tissue formation), and organogenesis (organ formation), all of which precede the emergence of a yolk-bearing embryo from the egg shell at hatch. Gross morphology and behavior are used to define those developmental milestones that occur after yolk absorption: free-to-move embryos before emergence from gravel nests, exogenously feeding alevin swimming free in the water, seaward migrating smolt, marine phase juvenile, and anadromous adult stages (reviewed for several species in Groot and Margolis 1991). But these attributes are not clearly quantifiable milestones.

Also, little information is available about post-hatch morphologic changes in yolk-bearing embryos. With the exception of yolk mass, no physical markers have been described that characterize the developmental stages that occur during the 2 to 7 month span between hatching and emergence (Malecha 2002), even though such descriptions might be used to identify stock structure, establish the developmental standards needed to make comparisons among strains of fish adapted to different environments or run timings, or make correlations between developmental landmarks and physiological events that occur during later stages. Given that gill raker counts are often used to characterize and identify species (Whitehead 1985; Humphries 1993; Yokogawa and Seki 1995), we expected that the progression of raker formation in embryos would serve as a useful post-hatch developmental marker. The typical pattern of post-hatch raker development has not been reported, nor is it known how this pattern is influenced by exposure to environmental variation or by increased genetic variability from hybridization.

The expression of traits that are used to characterize developmental stages typically show little variation in salmonid fish (Pelluet 1944; Ballard 1973). Such developmental stability is apparently canalized by homeostatic mechanisms that result in consistent phenotypes; the mechanisms buffer development against the disruptive effects of minor genetic and environmental perturbation (Waddington 1942). Even in populations that have substantial genetic diversity or that experience a wide range of environmental conditions, the phenotypic variability of critical morphological characteristics is low and individuals are similar to each other (Waddington 1957). Exposure to traumatic events deriving from genetic or environmental change, however,

can disrupt these homeostatic pathways and increase phenotypic variability (Valentine et al. 1973; Leary and Allendorf 1989; Clarke 1993).

The primary objective of this study was to evaluate the increasing number of gill rakers as a post-hatch developmental marker in Pacific salmon by observing development in fish exposed to genetic hybridization and environmental differences (temperatures above ambient) during early development. Observation of the variability of post-hatch gill raker development were made in (1) control fish – native pink salmon (*O. gorbuscha*) incubated in their native waters; (2) third-generation hybrids between spatially separated populations; and (3) native pink salmon reared in warmed water.

MATERIALS AND METHODS

Breeding and Incubation

Pink salmon were collected at Auke Creek Research Station (a facility of US National Oceanic and Atmospheric Administration's Alaska Fisheries Science Center) from a weir near the mouth of Auke Creek, a 350 m high-gradient, lake-fed stream located near Juneau, Alaska, a stream in which populations of pink salmon naturally spawn (Gilk et al. 2004). Mature salmon used in this experiment were fin-marked individuals that represented the second filial (F_2) generation of a breeding experiment involving native Auke Creek pink salmon and spatial hybrids that had been released as fry to the North Pacific Ocean from the Auke Creek Research Station during the spring of

2000. The hybrid line was originally created by hybridizing females in Auke Creek with males from Pillar Creek on Kodiak Island, Alaska (Gilk et al. 2004). Pillar Creek is a 1800 m long reservoir-fed stream located 1000 km directly west of Juneau that is historically 1 to 2 °C cooler than Auke Creek between late August when the salmon spawn and mid-November when embryos hatch. Although the latitudes (near 58° N) and habitats of Auke and Pillar Creek are similar, their spatial separation and different temperature regimes make it likely that the groups have evolved independently into genetically distinct and locally adapted populations (Adkison 1995; Gilk et al. 2004, Wang et al. 2007). Crosses between these stocks produced outbred hybrids that allowed us to determine if hybridization influenced gill raker development. Culture of the native population in natural and warmed water allowed us to determine if increased temperature affected gill raker development.

Maturing salmon were collected during late August of 2001 as they returned to Auke Creek, sorted according to gender and fin clip (native or hybrid), and held for several days until maturation was complete. On 8 September 2001, a blocked 2x2 factorial design was used to produce full- and half-sib F_3 families from native (32 males and 32 females) and hybrid (20 males and 20 females) returning fish. There were 16 blocks of natives for a total of 64 families and 10 blocks of hybrids for a total of 40 families.

Fertilized eggs were incubated in divided trays (FAL™; Marisource Milton, WA) that housed each family separately. Each tray had 10 compartments which contained single families. The trays were placed in incubation cabinets so that each cabinet

contained eggs from a single type of cross (native or hybrid). The eggs in one native and one hybrid cabinet were incubated in ambient temperature Auke Creek water. Because water temperatures in the stream, intragravel environment (redds), and research station are similar, development of natives reared under ambient conditions represented the normal development of wild Auke Creek pink salmon. These natives were the control group. A subset of eggs from each of the native families were placed in a third cabinet and incubated in a simulated environment that was, on average, 2.4 °C (SD ± 0.8 °C) warmer than ambient Auke Creek stream temperatures through hatching (Fig. 4.1).

Temperature in the simulated environment was altered daily to mimic natural variable incubation conditions (Fig. 4.1). Water in the warm regime was re-circulated to achieve the level of control needed to simulate daily thermal fluctuations in a captive setting. Two 1136-liter Living Streams® (Frigid Units, Inc. Toledo, OH) were used as a reservoir and equipped with a 1-hp chiller and two aquarium heaters. A ¾-hp submersible pump was used to move water from the reservoir to a 76-liter head tank, which distributed water to the incubation cabinets by a gravity feed. Water from the incubators drained into the reservoir, where it was conditioned and pumped back to the head tank. Water in the reservoirs was continually replaced with fresh stream water; a complete turnover occurred every 5 hours. After approximately 14 weeks of development, the heaters were removed and ambient temperature Auke Creek water was allowed to flow unidirectionally through the cabinets for the remainder of the experiment; after the heaters were removed, water temperatures in the two regimes were identical (Fig. 4.1). Although removal of the heaters occurred before the final three samples were collected,

we did not believe it substantially affected total raker formation because developmental patterns of vertebrate embryos are established early in development (Gilbert 2000).

Water was supplied to each incubator cabinet at a rate of 8 L/min until the eyed stage was reached, and at 23 L/min thereafter. Temperatures were recorded daily to the nearest 0.1 °C and did not differ substantially between incubation cabinets for a particular temperature regime. Before hatching, incubating embryos were treated once a week with formalin (1:6000 in static water) for one hour to reduce infestations by fungus and algae, a standard practice in the culture of salmon embryos. After hatching, weekly salt treatments (3 parts per thousand sodium chloride for 1 hour) were used to minimize infestations, another common practice in salmon culture. All water was filtered through sheer nylon mesh prior to entry into the incubation cabinets to prevent hydra infestation. Embryos were held until individuals in the native control group absorbed their yolk reserves, the stage at which salmon embryos naturally emerge as alevin from stream gravel and begin their exogenously-feeding free swimming lives.

Sampling and Raker Counts

One yolk-bearing embryo was collected from each of 5 randomly chosen native and hybrid families reared in ambient temperature water every 30 to 50 ATU (accumulated thermal units: a unit of measurement that quantifies thermal exposure over time; one ATU is equal to one degree Celsius for one day) to track the developmental progression of gill rakers in post-hatch individuals. One yolk-bearing embryo was also

collected from each of 5 native families incubated in warm water. These families were the same as those sampled from the ambient temperature incubators. The same native and hybrid families were sampled throughout the experiment to minimize any potential for confounding genetic effects. This sampling scheme produced three treatment groups: ambient natives (i.e. controls; AC), ambient hybrids (AH), and warm natives (WC).

Sampling began at 743.5 ATU, when rakers became visible on the first branchial arches of embryos in the warm regime, and continued until approximately 1,100 ATU for a total of nine sampling intervals (Fig. 4.1). Gill raker formation appeared to have stopped when yolk reserves were depleted, and we assumed that the number of rakers did not diminish during the short-term starvation that likely occurred at the end of the experiment. Embryos were fixed in 10% buffered formalin for approximately 24 hrs, transferred to distilled water for 24 hr, and stored in 40% isopropyl alcohol. The first branchial arches on the left and right side were excised with forceps under a dissecting microscope (10X magnification) and mounted in distilled water on a glass slide so that gill rakers could be observed. The first arch was chosen because it was easily removed, a factor that needs to be considered when developing a tool to provide a quick and efficient evaluation of developmental status. A transmitted light microscope equipped with a polarizing lens and a high-resolution video camera was used to capture images at a magnification of 40X so that the number of rakers on each gill arch could be counted (Fig. 4.2). Standard length (SL) and yolk weight were also recorded because both parameters have been used to define developmental stages. Fish samples were stored in

90% ethanol prior to dissection, which solidified the yolk into a single mass. Removal and measurement of this yolk mass allowed us to consistently estimate of yolk weight.

Statistical Analysis

We conducted Shapiro-Wilk and Kolmogorov-Smirnov tests with the PROC UNIVARIATE module in SAS (SAS Institute 2002) to determine if the raker counts and yolk weights were normally distributed within each cross and incubation regime. Because gill raker counts from the left and right arches were bilaterally symmetrical within cross and rearing environment throughout the study period (Restricted Maximum Likelihood; $p > 0.10$, Appendix A), counts from the two sides were added within treatments prior to comparison. Consolidated counts into a single term for each individual that preserved the overall variation associated with counts from each side of the fish.

Restricted maximum likelihood analysis (REML) was used to determine how hybridization and incubation temperature influenced gill raker development and yolk consumption. This method of variance analysis was used instead of the least squares analysis of variance because it is more robust to deviations from normality and non-homogenous variances (Shaw 1987: Searle et al. 1992; Lynch and Walsh 1998), both of which were associated with our data. Trends in gill raker development and yolk consumption were examined within each treatment group with a single-factor REML model:

$$Y_{jk} = \mu + T_j + \varepsilon_{jk} \quad (1)$$

where Y_{jk} was the dependent variable (e.g. the number of gill rakers per embryo or the amount of yolk in grams), T_j was the effect of ATU, and ε_{jk} was the residual random error. The ATU term was treated as a fixed (categorical) effect because sampling within crosses and across incubation regimes was standardized by ATU (temperature differentials were ≤ 0.9 °C), and each sampling event represented a discrete period of embryo development. Linear regressions were used to estimate the rate of raker formation and yolk consumption within each treatment.

A two-factor REML model was used to evaluate the effects of hybridization and temperature on raker development patterns and yolk consumption:

$$Y_{ijk} = \mu + G_i + T_j + G_i{*}T_j + \varepsilon_{ijk} \quad (2)$$

where Y_{ijk} was the dependent variable (e.g. the number of gill rakers per embryo or the amount of yolk in grams), G_i was the effect of treatment group (e.g. cross [AC and AH] or incubation regime [ambient and warm]), T_j was the affect of ATU, $G_i{*}T_j$ was the effect of the interaction between treatment group and ATU, and ε_{ijk} was the residual random error. Cross and ATU were treated as fixed (categorical) effects. An insignificant interaction term indicated that the effects of treatment group and ATU were independent.

Pairwise comparisons between treatments allowed us to determine if cross or temperature influenced development patterns. Bonferroni corrected post-hoc pairwise

comparisons were made along with each REML analysis to compare raker counts in consecutive sampling events within treatments and to identify discrete stages of raker development. All comparisons and hypothesis tests were conducted in SAS with PROC MIXED procedures. The significance level for hypothesis testing was set at $p \leq 0.05$. If gill raker development progressed in a temporally predictable fashion and was unaffected by hybridization and incubation temperature, we considered it to be developmentally stable and, therefore, potentially suitable for use as a post-hatch developmental marker.

RESULTS

Some of the data were not normally distributed (Shapiro-Wilk tests; $p \leq 0.01$). Noting that normality tests often return false positives when samples sizes are small (Palmer 1994), that skewness and kurtosis measures associated with each treatment and variable fell within the range of values characteristic of normal distributions: -0.89 to 0.89 and -1.79 to 1.79, respectively (Palmer 1994), and that square, and square root transformations did not substantially alter results of analyses and non-parametric tests yielded the same statistical conclusions as their parametric counterparts, we conclude that REML analyses were sufficiently robust to compensate for deviation from normality inherent in the raw data as a result of small sample sizes.

Gill raker counts were positively correlated with SL within each treatment group (Linear regressions; $p \leq 0.001$), with adjusted squared multiple R values that ranged between 0.65 and 0.83. Despite this correlation, we did not standardize raker counts by

length because SLs were similar between crosses incubated in ambient temperature stream waters, differing by only 1.0% ($F_{[1,72]} = 1.54, p = 0.219$). Embryo growth rates were also similar ($F_{[8,72]} = 0.26, p = 0.98$). Using these raw counts in our analyses allowed us to preserve the standard error, thereby providing a more accurate evaluation of developmental stability.

Development in Controls

The first step taken to evaluate the usefulness of gill rakers as a post-hatch developmental marker was to determine how rakers developed in native Auke Creek pink salmon reared at ambient stream temperatures (e.g. AC embryos). Overall trends in development indicated that the number of rakers in AC embryos increased as thermal units accumulated (Fig. 4.3). When sampling began, combined counts from the left and right arches averaged 23.20 (SD ± 1.64) rakers per fish. Their subsequent development during the next 5 sampling intervals occurred at a rate of 0.10 rakers per ATU ($R^2 = 0.93$) and was positively correlated with ATU ($F_{[8,36]} = 56.10, p < 0.001$). After 965.8 ATU, however, development stopped and counts reached a maximum of 39.55 (SD ± 1.76) rakers per embryo (Fig. 4.3).

For rakers to be useful as a developmental landmark, distinct changes in raker numbers must be observed. Pairwise comparisons indicated that raker counts increased in each consecutive sample during the first 5 sampling events ($p \leq 0.030$; Fig. 4.3). Raker

counts among the remaining samples were similar ($p > 0.999$) because raker development had reached a maximum.

Yolk consumption among native embryos were opposite of trends observed for raker development; yolk weight decreased as ATU increased (Fig. 4.4). When sampling began, AC embryos had 42 (SD ± 15) mg of yolk. Subsequent yolk abundance was negatively correlated with ATU ($F_{[8,36]} = 29.32$, $p < 0.001$), and it was metabolized at a rate of 0.20 mg per ATU ($R^2 = 0.67$) through the first five sampling intervals (Fig. 4.4). Yolk reserves became internalized (e.g. ventral suture closed) at 852.6 ATU and were completely consumed after 965.8 ATU, which coincided with maximum raker counts (Fig. 4.4).

Effect of Hybridization on Development

Developmental trends in native and hybrid embryos were compared in ambient temperature to determine if hybridization influenced gill raker formation or yolk consumption. Raker development in hybrids incubated at ambient temperatures was similar to that of native embryos reared in the same environment (Fig. 4.3). Although REML analysis indicated that total raker counts differed between crosses ($F_{[1,72]} = 11.64$, $p < 0.001$), the difference in counts during each discrete sampling event was small; hybrids had one to three fewer rakers than natives during each sampling interval. Post-hoc pairwise comparisons confirmed that raker counts made at the same ATU were always equivalent and that the developmental progression of rakers was similar between

crosses ($p > 0.999$; Fig. 4.3). The interaction term was not significant ($F_{[8,72]} = 0.44, p = 0.896$), which indicated that there was no nonlinear effect of cross and ATU on raker formation.

Yolk consumption was also unaffected by hybridization (Fig. 4.4). Yolk was consumed at similar rates in each cross during the course of the experiment (REML; $F_{[1,72]} = 0.02, p = 0.876$). Cross and ATU did not interact to influence yolk metabolism ($F_{[8,72]} = 0.52, p = 0.839$).

Because no significant differences in raker development, raker symmetry, yolk consumption, or standard length (28.4 ± 1.9 mm and 28.7 ± 1.6 mm for natives and hybrids, respectively) were detected between native and hybrid fish that were incubated at ambient temperature, raker counts and yolk weights from these treatments were pooled to increase statistical power prior to comparison with fish reared in the warm regime.

Effect of Temperature on Development

To determine how increased temperature influenced developmental patterns, developmental trends of native and hybrid embryos reared at ambient temperatures were compared to those of embryos which had been incubated in warmer water (e.g. WC embryos). Sampling, however, had been standardized across incubation regimes by sampling in each at the same ATU, which meant that the standardized data lacked a temporal component. Consequently, the results were examined both in terms of ATUs and days.

At the same ATUs, embryos from the warmed incubators had 7 to 12 fewer rakers than those reared at ambient temperatures during all but the last sampling interval ($F_{[1,117]} = 386.07, p \leq 0.001$), and pairwise comparisons indicated that developmental trajectories differed between incubators ($p = 0.032$), primarily because raker formation in AC and AH embryos reached a maximum, whereas development in WC embryos did not (Fig. 4.5A). By the end of the experiment, however, counts in both regimes were similar, averaging nearly 40 rakers per embryo ($p > 0.999$; Fig. 4.5A). This development was positively correlated with ATU in both environments. Because thermal accumulation rate depended on incubation temperature and temperature influenced raker development, the interaction between ATU and incubation regime was significant ($F_{[8,117]} = 12.97, p \leq 0.001$).

Yolk consumption also differed between temperature regimes (Fig. 4.6A). Although yolk consumption in both incubators was positively correlated with ATU, embryos from warmer water had 3 to 11 mg more yolk during each sampling interval than those from the ambient regime ($F_{[1,117]} = 335.62, p \leq 0.001$). Pairwise comparisons indicated that the pattern of its consumption differed between incubators ($p < 0.001$). Native embryos incubated in the warmer water had yolk remaining at the conclusion of the experiment, whereas embryos incubated at ambient temperatures had metabolized all of their reserves mid-way through the sampling regimen (Fig. 4.6A).

When compared strictly on a temporal basis, the number of gill rakers accumulated much faster in the embryos exposed to the warmer temperature. At the beginning of the experiment (750 ATU), the WC embryos averaged 7.0 fewer rakers than

their ambient counterparts, but they were collected 55 days earlier (Fig. 4.5A). Their subsequent developmental maximum, however, was equivalent to that of the ambient embryos, and this maximum was attained 30 days earlier than that of the AC embryos, but with an exposure to 139 more ATUs (Fig. 4.5B). Linear regression also indicated that warmer water accelerated raker development. The WC embryos formed 0.39 rakers per day ($R^2 = 0.80$) when the water was heated, whereas embryos in ambient temperature water accrued 0.16 rakers per day ($R^2 = 0.88$) during the corresponding sampling period (Fig 4.5B). The rate of thermal accumulation for WC embryos declined considerably once the heaters were removed (Fig. 4.1), and there was a commensurate decline in raker development (0.14 rakers per day, $R^2 = 0.83$, Fig. 4.5B).

Because raker development is correlated with yolk availability, temperature had a similar effect on yolk consumption. The yolk mass of embryos reared at ambient temperatures became internalized after 184 days, whereas embryos in warm water reached the same milestone 32 days earlier after exposure to 187 more ATU (Fig. 4.6B). While the heaters were engaged, WC embryos consumed 1.66 mg of yolk per day ($R^2 = 0.60$) whereas, embryos incubated at lower ambient temperatures used 0.37 mg per day ($R^2 = 0.66$) over the same period (Fig. 4.6B). After the heaters were removed, there was a commensurate decline in yolk consumption among WC embryos (0.19 mg per day, $R^2 = 0.34$; Fig. 4.6B).

There were some indicators that the accelerated rate of yolk absorption and raker development had little effect on raker counts because the number of rakers associated with a specific amount of yolk was similar in each treatment regardless of when the fish

was collected (Fig. 4.7). For example, embryos from ambient and warm incubators that contained 29 mg of yolk both had 28 rakers, even though their collection dates were 40 days apart. Similarly, embryos with about 46 mg of yolk had approximately 23 rakers, regardless of treatment and sampling time. Additional comparisons could not be made because the amount of yolk present in the remaining samples was not equivalent between treatments.

DISCUSSION

There were several indications that the developmental progression of gill rakers can reliably be used as a post-hatch developmental marker in pink salmon for the period of development between hatching and exogenous feeding. Gill raker formation was incomplete at hatch and their subsequent development followed a sequential and predictable pattern. At hatch, the total number of rakers on the first left and right branchial arches of native embryos incubated at ambient temperatures averaged 23 rakers per fish. Subsequent development was nearly linear over time until counts peaked at 40 rakers 15 weeks later when yolk reserves had been consumed. In contrast, adult pink salmon throughout their range have between 48 – 70 rakers on the first arches (Miller and Lea 1972); and adults from Auke Creek typically have a total of 60 rakers (Gharrett and Smoker 1991). Clearly, raker formation was not complete by the end of this experiment; its development likely ceased as a result of starvation.

Another indication that gill rakers would make good developmental markers is that their formation is developmentally stable and consistent. The pattern of yolk consumption and gill raker development observed among hybrids incubated at ambient temperatures was nearly identical to that observed among natives reared in the same conditions, which indicated that raker development was buffered against the negative effects of hybridization. Because our hybrids were three generations (F_3) removed from the hybridization of two geographically isolated and locally adapted stocks, our comparison evaluated the effects of a specific type of hybridization known as outbreeding. Outbreeding depression can occur when genetically dissimilar groups interbreed, which can disrupt locally adapted gene complexes thereby reducing genetic health and fitness (Shields 1982) and altering developmental pathways and phenotypic expression (Marshall and Spalton 2000; McClelland et al. 2005). In Auke Creek pink salmon, outbreeding has been shown to decrease survival among F_2 spatial hybrids of the same line that we observed (Gilk et al. 2004), so it is likely outbreeding would have influenced the F_3 generation used in this experiment. Raker development, however, was apparently unaffected by such influences; perhaps because it is strongly canalized or because there is little genetic variation in this trait, which may stem from its importance to fitness (Moller 1997; Debat and David 2001; Gaillard and Yoccoz 2003).

Environmental factors can influence fish development (Valentine et al. 1973; Campbell et al. 1998; Campbell 2003), but comparisons of yolk consumption and raker growth between incubation regimes were confounded by differences in water temperature. Our sampling design was standardized by ATU because thermal units

accrued more rapidly in the warm incubators; and WC embryos achieved ATUs targeted for sampling before their ambient counterparts. As a consequence, WC embryos usually had more yolk and fewer gill rakers than the corresponding samples from the ambient regime that were collected 8 to 16 weeks later. Standardizing data by ATU also over-simplified the interaction between incubation temperature and development rate by eliminating temporal information. This made some graphs appear as if elevated incubation temperatures slowed embryo development (Fig. 4.5A and 4.6A).

In contrast to comparisons based on ATU, it was evident that warmer water substantially accelerated development rate when comparisons were made within a temporal context. Embryos reared in warmer water began to form rakers on the first gill arches 8 weeks before those incubated in cooler ambient waters. Although the raker count at the beginning of the experiment (750 ATUs) was lower in WC embryos than in the AC and AH embryos, the maximum accumulated count was the same in both temperature regimes but occurred a month earlier with yolk reserves to spare among embryos in the warm regime. Embryos incubated at ambient temperature used all their yolk to achieve the same developmental milestone. Moreover, the rate of gill raker development is not strictly a function of ATU accumulation. The developmental progression was positively correlated with temperature: faster accumulations of thermal units were associated with increased rates of yolk use and raker development, which is to be expected given the ectothermic nature of fish metabolism (Jobling 1994; Ban 2000; Bestgen 2008). The number of rakers associated with a given amount of yolk, however, was similar

regardless of incubation temperature, indicating that neither yolk consumption rate nor development rate affected raker counts.

Additional insight regarding the stability of gill raker development was discovered by examining its symmetry. In bilaterally symmetrical organisms, genetic and environmentally induced stress can cause developmental instability that can be expressed as physical asymmetry (Parsons 1990; Palmer and Strobeck 2003; Johnson et al. 2004). In this study, gill rakers exhibited persistent bilateral symmetry after being subjected to captivity, outbreeding, and atypically high incubation temperatures, thereby providing further support for the canalized nature of raker development. This symmetry means it would be possible to use raker counts from a single arch to characterize developmental stages.

Gill raker development is predictable and appears to be sufficiently stable to serve as a reliable post-hatch developmental marker in pink salmon for the period of development between hatching and exogenous feeding. Such a marker could be used to provide standards and accuracy for developmental studies, identify stock structure, and facilitate communication among aquaculturalists and biologists. Rakers were easily obtained, observed and enumerated, grew in a predictable sequence, and were developmentally stable in the face of both genetic (outbreeding) and thermal perturbation. Temperature influenced development rate, but it did not substantially influence total raker counts. Developmental patterns in our captive stocks were similar to those reported for their wild counterparts (Gharrett et al. 1999; Taylor and Lum 2003), so our observations likely represent natural patterns. Additional research, however, is advisable to provide

definitive conclusions regarding their usefulness as post-hatch developmental markers. Specifically, larger samples sizes and more frequent sampling will provide an improved evaluation of their developmental stability, as well as to determine the sampling intervals that are appropriate to adequately identify and define discrete stages of gill raker development. A more thorough assessment regarding the affects of changes in development rate on raker counts should also be considered. Because zooplankton size can influence raker development (Witte et al. 2008), intraspecific variation in raker development needs to be evaluated. Lastly, raker development in other fish should be examined to determine if rakers develop in a similar manner among other species. Although the widespread distribution of pink salmon throughout the North Pacific Ocean and its marginal seas (reviewed by Heard 1991) makes it a good model for evaluating the potential applicability of rakers as a post-hatch developmental marker for salmonids, these results may not be applicable to other fishes.

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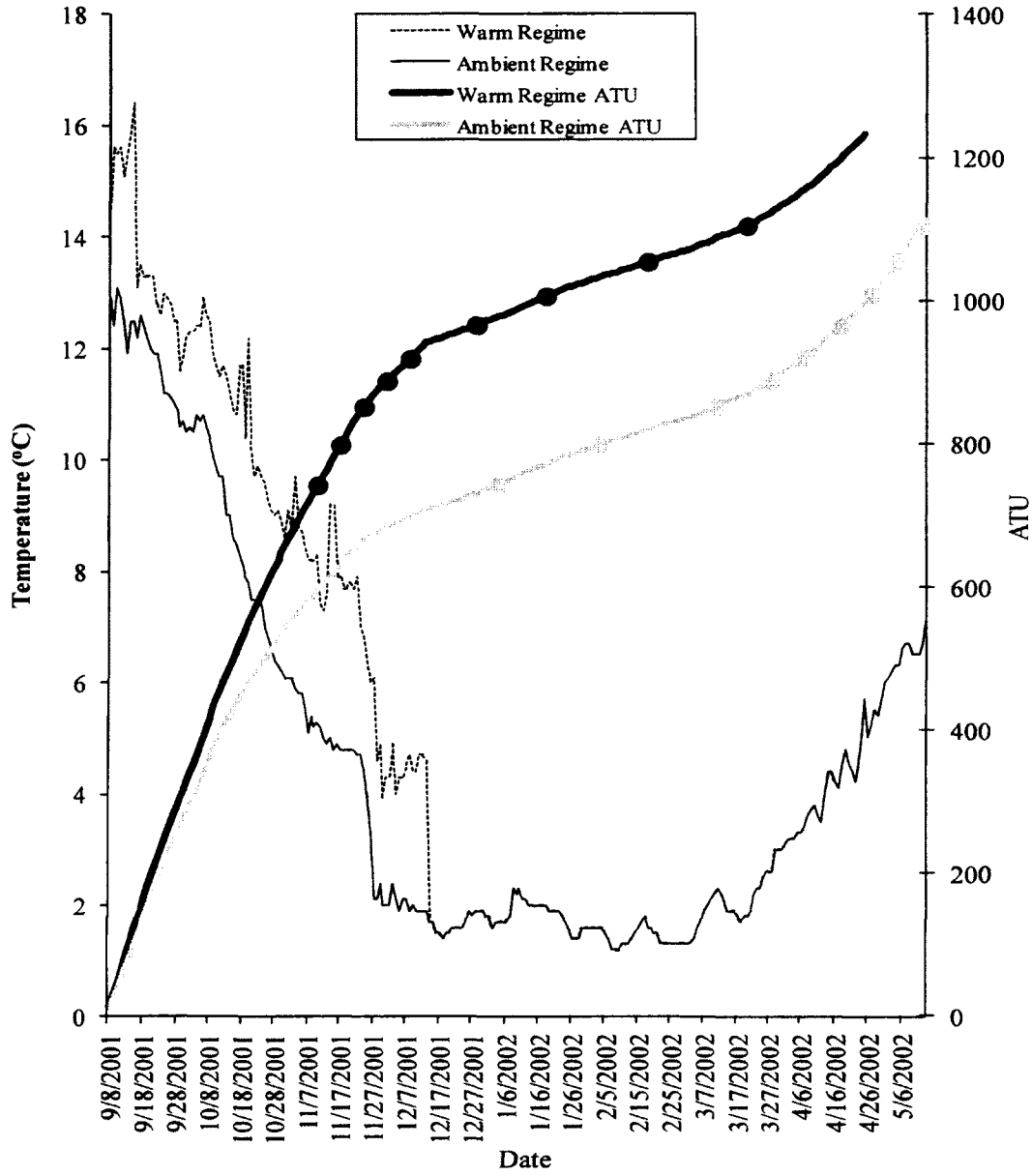

Figure 4.1. Water temperatures and accumulated thermal units (ATU) for two rearing environments for brood year 2001 pink salmon reared at Auke Creek Hatchery near Juneau, Alaska from 9/8/01 through 5/13/02. Ambient temperatures represent Auke Creek stream temperatures, whereas the heated regime was designed to mimic the natural variation of a warmer incubation environment. The black and grey circles indicate sampling intervals for the warm and ambient incubation environments, respectively. Water heaters were taken offline on 12/13/01.

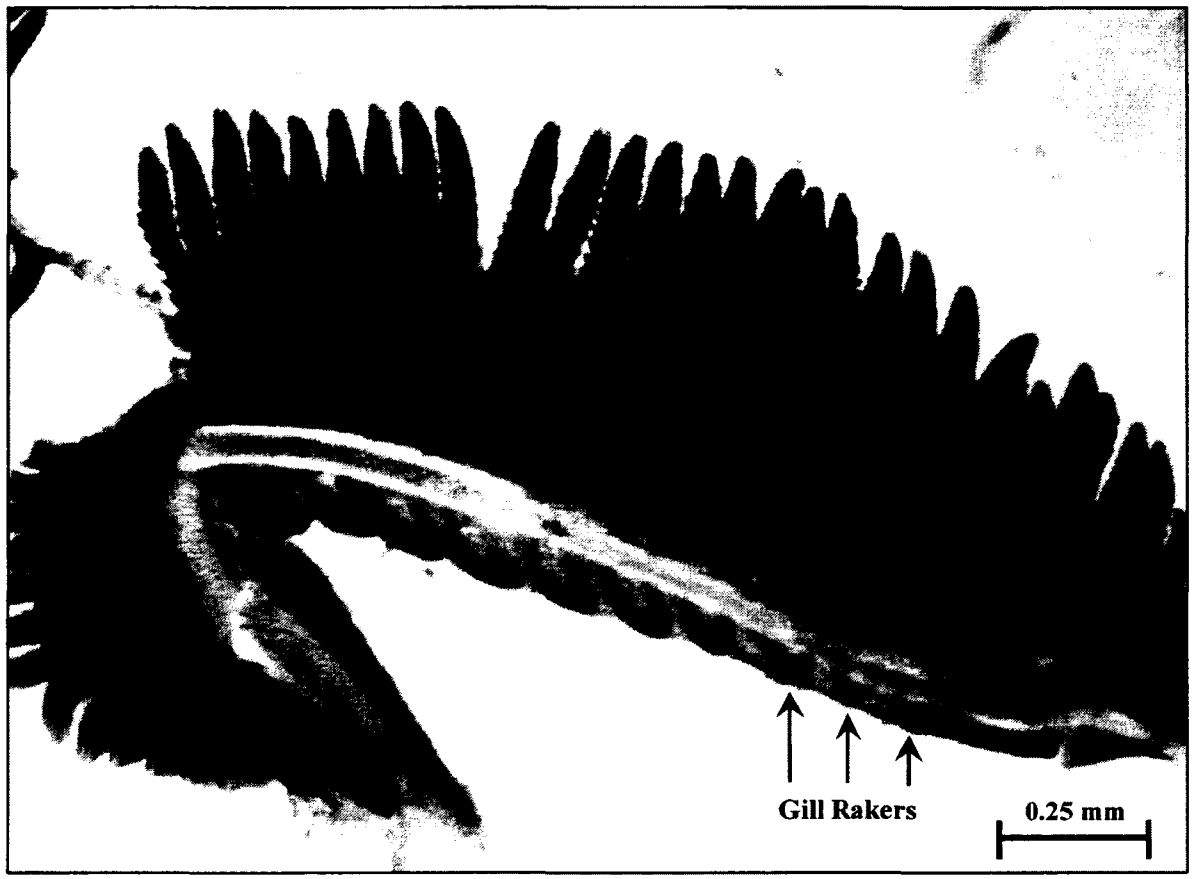

Figure 4.2. The first branchial arch from the left side of a pink salmon embryo removed after 118 days (743.5 cumulative thermal units) of development.

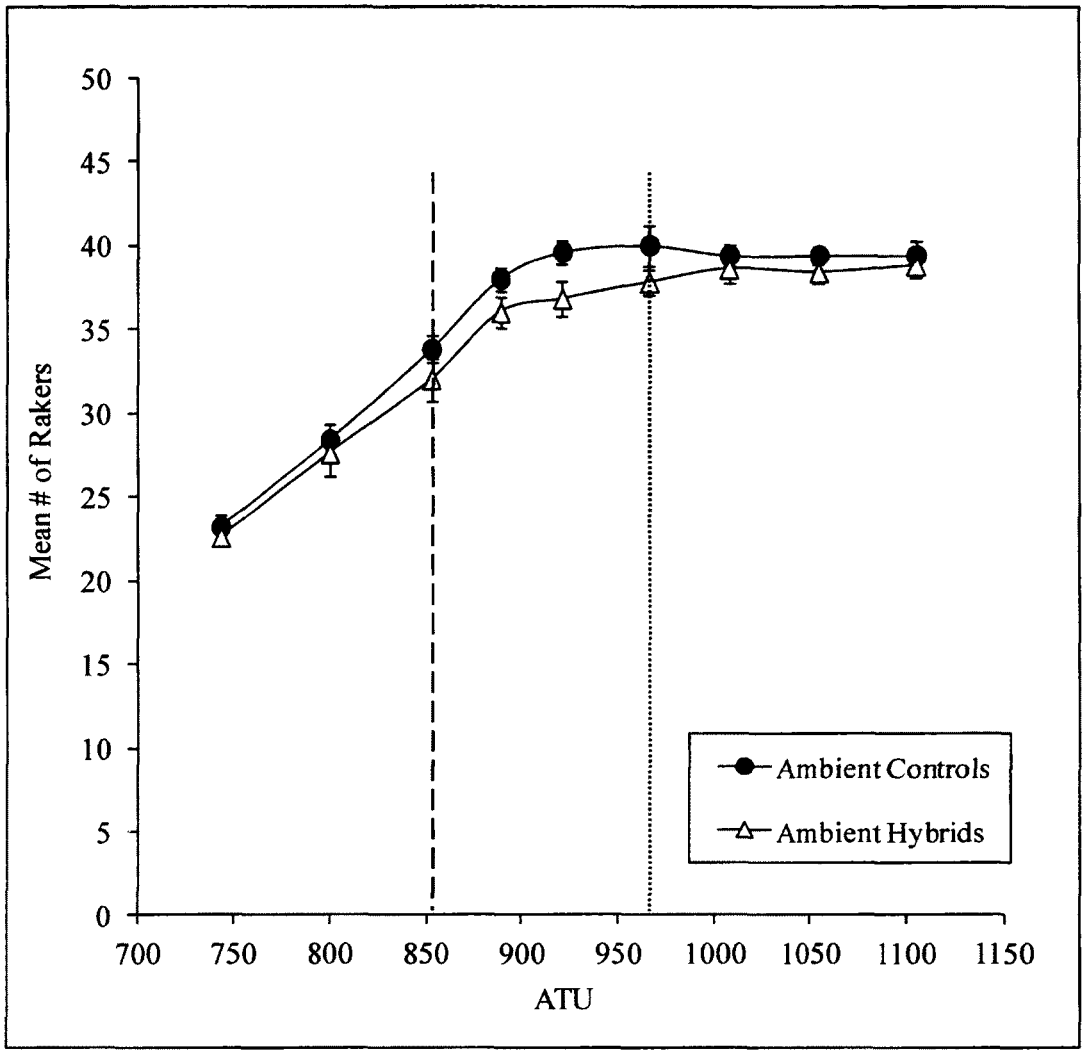

Figure 4.3. The effect of hybridization on the developmental progression of the total number of gill rakers from the first left and right branchial arches relative to accumulated thermal units (ATU) in pink salmon embryos incubated in ambient temperature Auke Creek waters. The dashed line indicates the ATU at which the yolk sac became internalized (e.g. "button-up") and the dotted line indicates when yolk was completely consumed. Error bars represent standard errors.

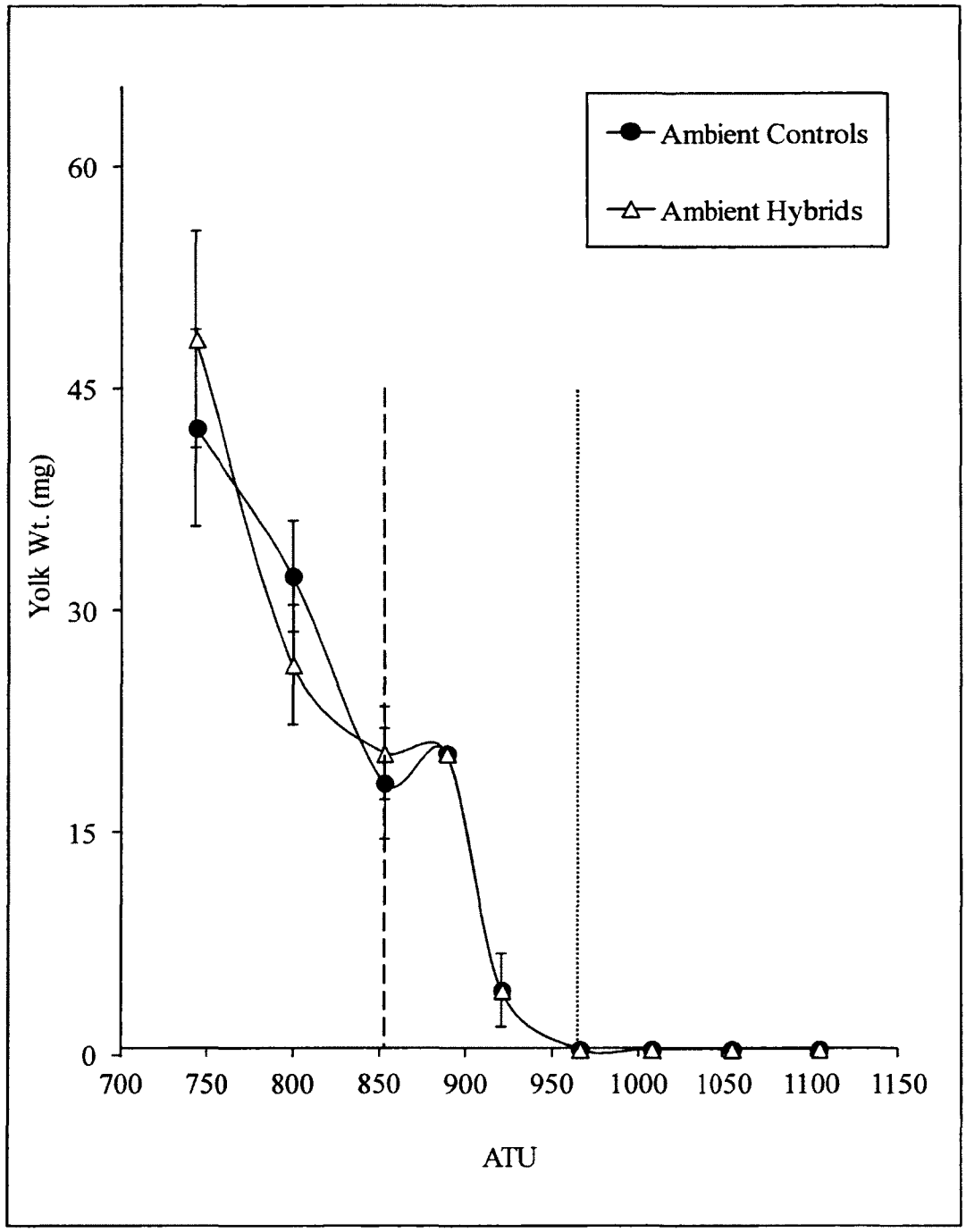

Figure 4.4. The effect of hybridization on yolk consumption relative to accumulated thermal units (ATU) in pink salmon embryos incubated at ambient Auke Creek stream temperatures. The dashed line indicates the ATU at which the yolk sac became internalized (e.g. "button-up") and the dotted line indicates when yolk was completely consumed. Error bars represent standard errors.

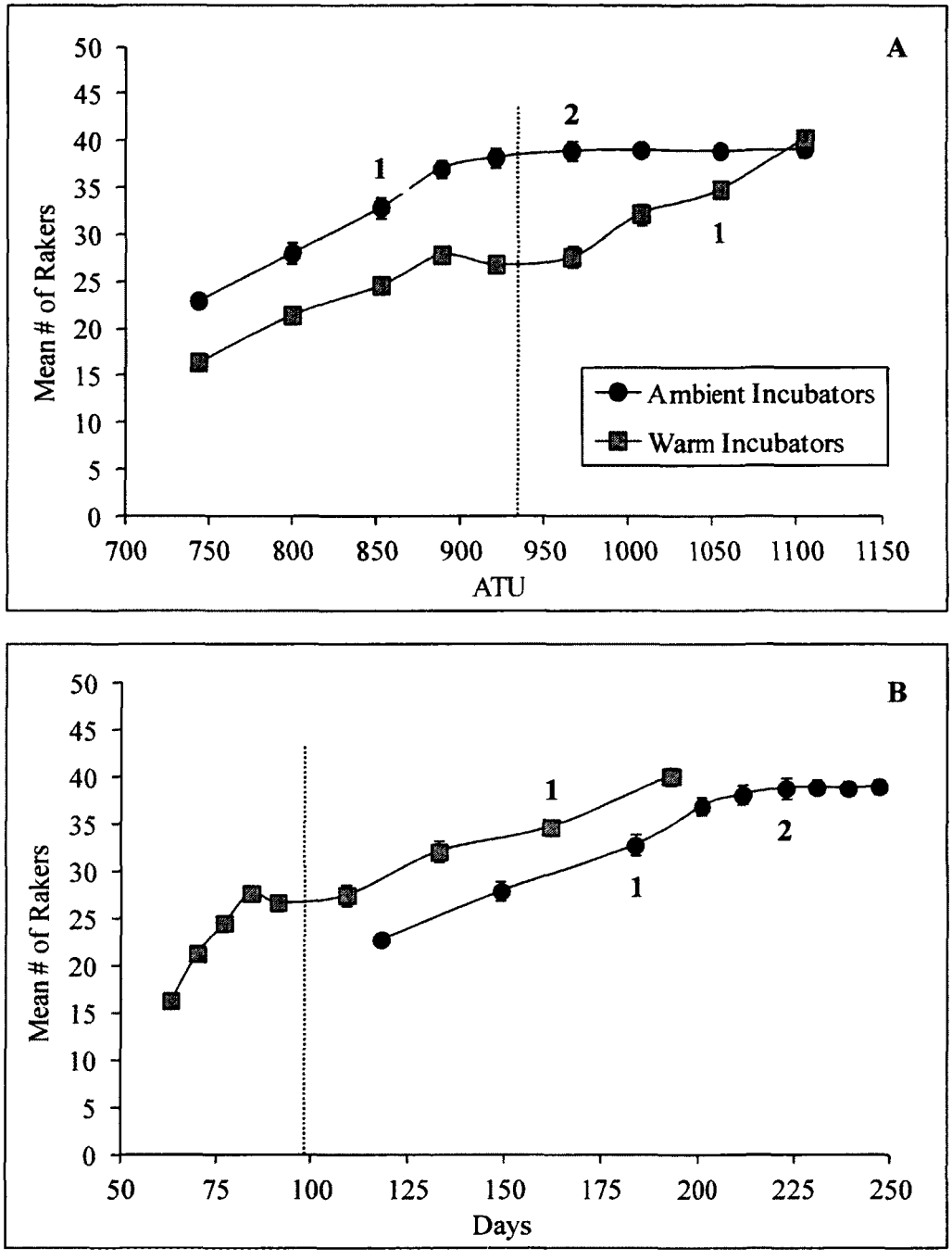

Figure 4.5. The effect of temperature on the developmental progression of gill rakers from the first left and right branchial arches relative to accumulated thermal units (ATU: A) and days (B) in pink salmon reared at ambient and elevated Auke Creek stream temperatures. The "1" indicates when the yolk sac became internalized (e.g. "button-up") and the "2" indicates when yolk was completely consumed. The dashed line indicates when the water heaters were taken offline. Error bars represent standard errors.

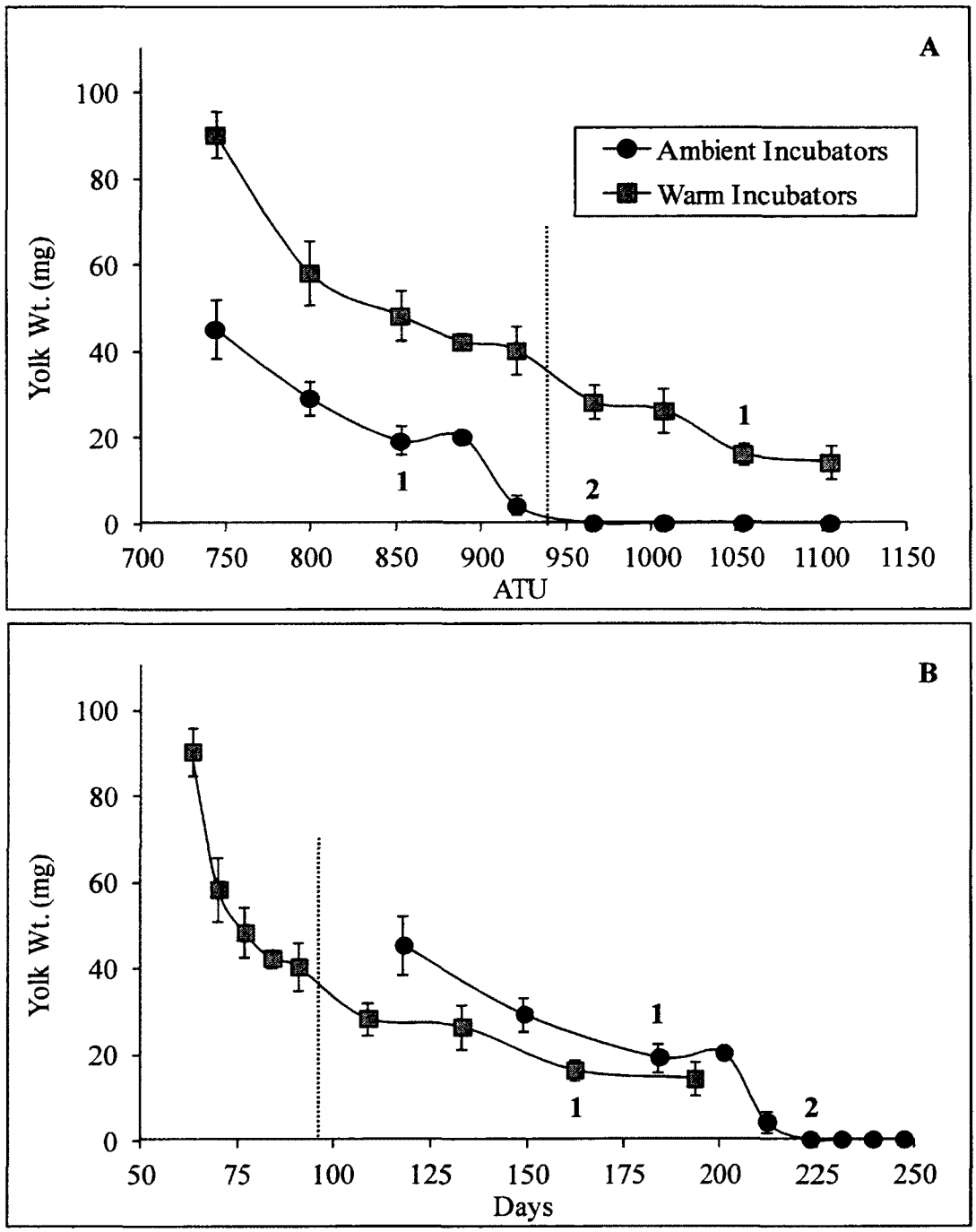

Figure 4.6. Effect of temperature on yolk consumption relative to accumulated thermal units (ATU: A) and days (B) in pink salmon incubated in ambient and elevated Auke Creek water temperatures. Yolk weights from natives and hybrids reared in ambient temperatures were pooled. The "1" indicates when the yolk sac became internalized and the "2" indicates when yolk was completely consumed. The dashed line indicates when the water heaters were taken offline. Error bars represent standard errors.

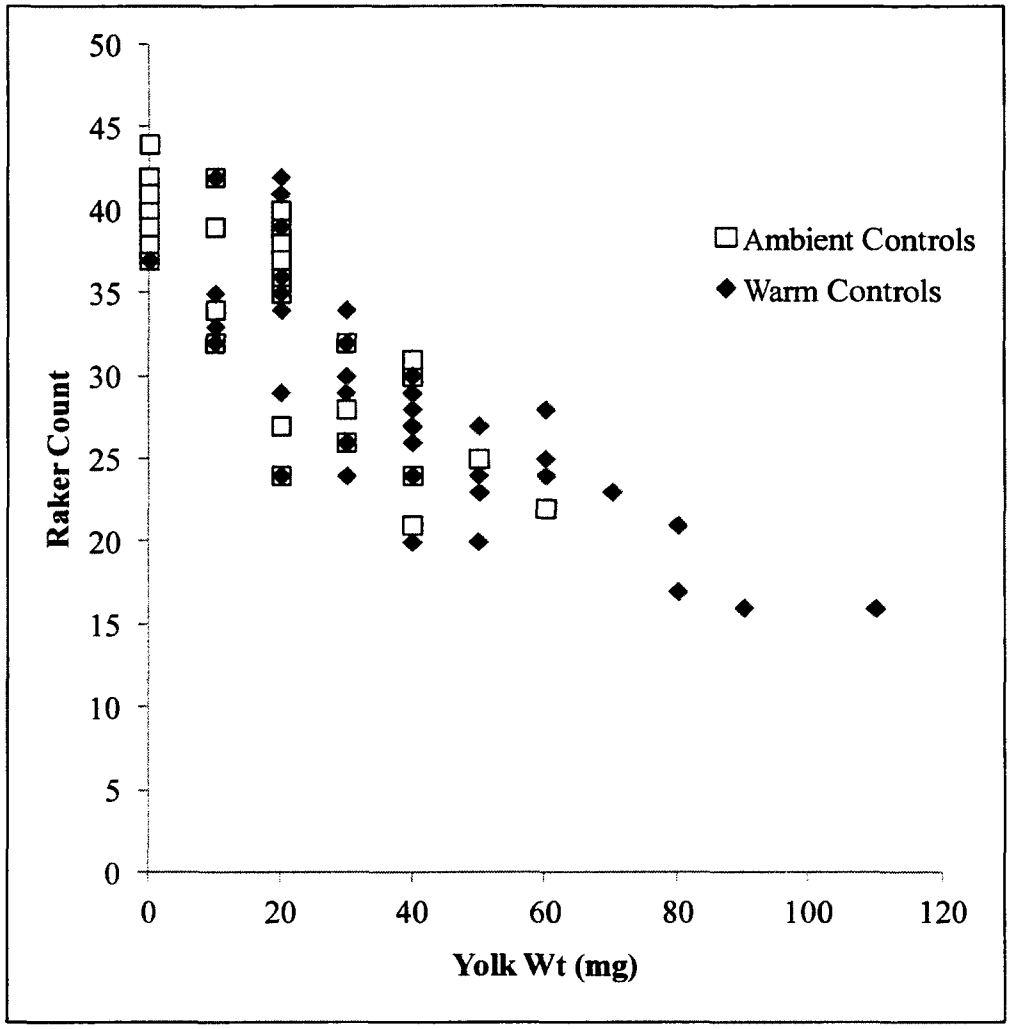

Figure 4.7. Relationship between yolk weight and total gill raker counts for native pink salmon reared in ambient and elevated Auke Creek water temperatures.

APPENDIX 4.A
Bilateral Symmetry of Gill Rakers

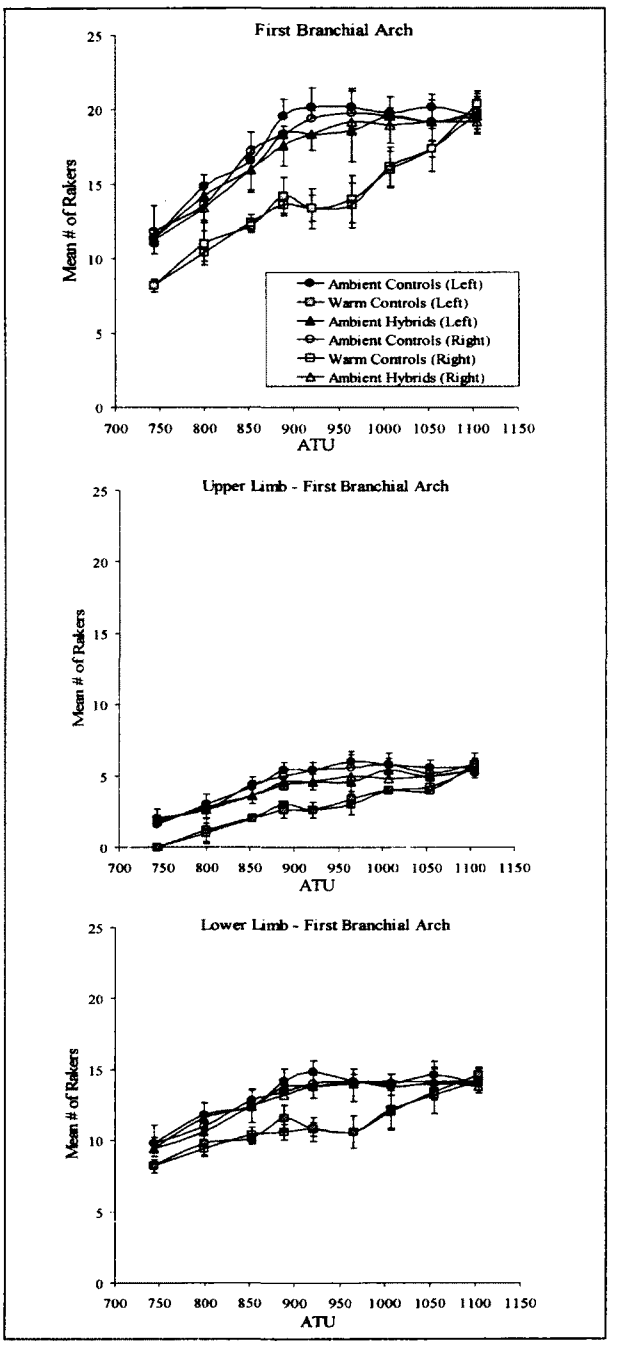

Figure 4.A. Bilateral symmetry of gill rakers on the entire, upper and lower arm of the first branchial arch relative to accumulated thermal units (ATU) from the left and right sides of native and hybrid pink salmon incubated in ambient temperatures, and natives reared at warmer temperatures. Error bars represent standard deviations.

APPENDIX 4.B
Result Tables for REML Analyses and Pair-wise Comparisons

Table 4.B-1. Two factor REML testing the effect of cross and ATU on standard length and growth rate of fish reared at ambient temperatures. The model is fixed and balanced.

Effect	Numerator df	Denominator df	F - Value	Pr > F
Cross	1	72	1.535	0.219
ATU	8	72	16.592	0.001
ATU*Cross	8	72	0.261	0.976

Table 4.B-2. Single factor REML and pairwise comparisons (*p* values) testing the effect of ATU on gill raker number in AC embryo. The model is fixed and balanced.

Effect	Numerator df	Denominator df	F - Value	Pr > F
ATU	8	36	56.099	0.001

ATU*	743.5	799.8	852.6	888.7	920.8	965.8	1007.3	1054.8	1104.8
743.5	1.000								
799.8	0.002	1.000							
852.6	0.000	0.001	1.000						
888.7	0.000	0.000	0.028	1.000					
920.8	0.000	0.000	0.000	1.000	1.000				
965.8	0.000	0.000	0.000	1.000	1.000	1.000			
1007.3	0.000	0.000	0.001	1.000	1.000	1.000	1.000		
1054.8	0.000	0.000	0.001	1.000	1.000	1.000	1.000	1.000	
1104.8	0.000	0.000	0.001	1.000	1.000	1.000	1.000	1.000	1.000

Table 4.B-3. Single factor REML testing the effect of ATU on yolk consumption in AC embryo. The model is fixed and balanced.

Effect	Numerator df	Denominator df	F - Value	Pr > F
ATU	8	36	29.321	0.001

Table 4.B-4. Two factor REML and pairwise comparisons (*p* values) testing the effect of hybridization on gill rakers in embryo reared at ambient temperatures. The model is fixed and balanced.

Effect	Numerator df	Denominator df	F - Value	Pr > F
Cross	1	72	11.639	0.001
ATU	8	72	91.481	0.001
ATU*Cross	8	72	0.435	0.896

ATU*	743.5	799.8	852.6	888.7	920.8	965.8	1007.3	1054.8	1104.8
743.5	1.000								
799.8	0.100	1.000							
852.6	0.000	0.710	1.000						
888.7	0.000	0.000	1.000	1.000					
920.8	0.000	0.000	1.000	1.000	1.000				
965.8	0.000	0.000	0.270	1.000	1.000	1.000			
1007.3	0.000	0.000	0.030	1.000	1.000	1.000	1.000		
1054.8	0.000	0.000	0.060	1.000	1.000	1.000	1.000	1.000	
1104.8	0.000	0.000	0.020	1.000	1.000	1.000	1.000	1.000	1.000

* Columns = Natives. Rows = Hybrids

Table 4.B-5. Two factor REML testing the effect of hybridization on yolk consumption in embryo reared at ambient temperatures. The model is fixed and balanced.

Effect	Numerator df	Denominator df	F - Value	Pr > F
Cross	1	72	0.024	0.876
ATU	8	72	58.226	0.001
ATU*Cross	8	72	0.518	0.839

Table 4.B-6. Two factor REML and pairwise comparisons (p values) testing the effect of incubation temperature on gill raker number. The model is fixed & unbalanced.

Effect	Numerator df	Denominator df	F - Value	Pr > F
Temperature	1	117	386.07	0.001
ATU	8	117	135.68	0.001
ATU*Cross	8	117	12.968	0.001

ATU*	743.5	799.8	852.6	888.7	920.8	965.8	1007.3	1054.8	1104.8
743.5	0.000								
799.8	1.000	0.000							
852.6	1.000	0.292	0.000						
888.7	0.002	1.000	0.001	0.000					
920.8	0.062	1.000	0.000	0.000	0.000				
965.8	0.004	1.000	0.000	0.000	0.000	0.000			
1007.3	0.000	0.023	1.000	0.003	0.000	0.000	0.000		
1054.8	0.000	0.000	1.000	1.000	0.292	0.032	0.023	0.032	
1104.8	0.000	0.000	0.000	0.521	1.000	1.000	1.000	1.000	1.000

* Columns = Ambient. Rows = Warm

Table 4.B-7. Two factor REML and pairwise comparisons (p values) testing the effect of incubation temperature on yolk consumption. The model is fixed and unbalanced.

Effect	Numerator df	Denominator df	F - Value	Pr > F
Temperature	1	117	335.624	0.001
ATU	8	117	78.513	0.001
ATU*Cross	8	117	4.589	0.001

ATU*	743.5	799.8	852.6	888.7	920.8	965.8	1007.3	1054.8	1104.8
743.5	0.000								
799.8	0.650	0.000							
852.6	1.000	0.006	0.000						
888.7	1.000	0.650	0.000	0.000					
920.8	1.000	1.000	0.001	0.003	0.000				
965.8	0.034	1.000	1.000	1.000	0.000	0.000			
1007.3	0.006	1.000	1.000	1.000	0.000	0.000	0.000		
1054.8	0.000	0.650	1.000	1.000	1.000	0.074	0.074	0.000	
1104.8	0.000	0.159	1.000	1.000	1.000	0.327	0.327	0.327	0.001

* Columns = Ambient. Rows = Warm

GENERAL CONCLUSIONS

In the preceding chapters, I presented the results of research that explored how environmental and genetic factors influenced development in pink salmon. In the first chapter, I examined how development time was influenced by inheritance, incubation temperature, and outbreeding. Development time in embryos from the odd-year broodline was influenced primarily by additive genetic factors, whereas it was genetically conserved in the even-year run. No significant genotype-by-environment (GxE) influences were observed in either broodline, indicating that the observed variation in development rate was likely the result of phenotypic plasticity, not local adaptation. The development time in a third generation of outbred hybrids indicated that outbreeding prolonged development in both broodlines and altered the proportions of additive and environmental variation, possibly by influencing the canalization process. These results complement previous studies that observed outbreeding depression in earlier generations of outbred pink salmon (Gharrett and Smoker 1991, Gilk et al. 2004, Wang et al. 2007).

This experiment documented the occurrence of outbreeding depression in pink salmon, indicated that the introgression of nonnative fish may erode fitness by altering locally adapted traits, and demonstrated that its effects can last at least three generations. The long lasting impact of outbreeding suggests that salmon populations should not be treated as a single homogeneous population and indicates that caution must be taken when making management decisions that could result in outbreeding between wild and cultured populations if we are to maintain salmon fitness and productivity.

In Chapter two, I explored how inheritance, environment, and outbreeding influenced otolith formation. Early otolith development was genetically conserved and canalized, and there was no evidence of local adaptation or outbreeding depression, but the phenotypic expression of these genes is plastic and strongly influenced by environmental factors. The distinction between genetically and environmentally derived variation is important when intraspecific differences in otolith structure is used to reconstruct life histories and identify stock structure. In this study, changes in otolith structure related to environmental variation were larger, more obvious, and more variable than those related to genetic variability. Genetically-induced variation was small probably because the genes associated with otolith formation were conserved, and sensitivity to environmental change did not vary among genotypes, or because the process is strongly canalized. Consequently, if the majority of phenotypic variation in otolith structure is caused by environmental influences, then stock structure based on such variation may be more an indication that groups of fish within a species were exposed to different environmental conditions than it is an indication of genetic differences. Although our results may not apply to all species, it indicates that intraspecific variation in otolith morphology should be compared among groups with caution when it is used to evaluate population dynamics.

Because environmental stress and hybridization can adversely affect development, in Chapter three I used fluctuating asymmetry in otoliths to evaluate the impact of decreased incubation temperature and outbreeding on developmental stability. Otoliths from pink salmon embryos incubated at decreased temperatures were bilaterally

asymmetrical, whereas the otoliths from outbred fish were not, although they exhibited evidence of increased phenotypic variation and heterosis. While this suggests that otolith asymmetry may be useful for the detection of environmental stress in pink salmon, its usefulness for detecting the effects of genetic perturbations is uncertain and warrants further evaluation. When these results are considered within the context of studies of otoliths from other species, it is evident that otoliths do not respond to stress the same way in all fishes (Alados et al. 1993, Panfili et al. 2005). Similarly, various aspects of otolith morphology can react differently to stress within a species (Green and Lochmann 2005). This lack of consistency within and among species suggests an otolith's developmental response to stress may be trait and/or species-specific. Consequently, an otolith's developmental response to stress should be thoroughly evaluated on a case by case basis before it can be used to evaluate developmental stability.

In the final chapter, I evaluated the usefulness of gill rakers as a post-hatch developmental marker for the period of development between hatching and exogenous feeding by tracking their formation in native, thermally stressed, and hybrid yolk-bearing pink salmon embryos. The results indicated that gill rakers could serve as reliable post-hatch developmental markers because they were easily obtained, observed and counted, grew in a predictable sequence, and were developmentally stable in the face of both genetic and environmental stress. Such a marker could be used to provide standards and accuracy for developmental studies, identify stock structure, and facilitate communication among aquaculturalists and biologists. Additional research, however, is advisable. Larger samples sizes and more frequent sampling are needed to provide an

improved evaluation of developmental stability, as well as to determine the sampling intervals that are appropriate to adequately identify and define discrete stages of gill raker development. Although the widespread distribution of pink salmon throughout the North Pacific Ocean and its marginal seas makes it a good model for evaluating the potential applicability of rakers as a post-hatch developmental marker for salmonids, these results may not be applicable to other fishes.

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